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ERRATUM

Page 157, 16th line from the bottom: for: "according to which the entrance of organic phosphate into . . ." read: "according to which the entrance of inorganic phosphate into . . .".

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STUDIES ON THE CYCLE OF ELEMENTS IN FRESH WATER

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CHAPTER I

INTRODUCTION

In this paper a report is given of an investigation into the chemical cycle in dutch fresh water lakes. In the biocoenosis in these lakes two cycles occur, that of the organisms and that of the chemical elements. Both cycles are very closely interwoven.

In the cycle of organisms the algae occupy a primary position. The existence of all subsequent heterotrophic organisms in the cycle depends fundamentally on the consumption of the autotrophic ones. We here make a differentiation between consumers – organisms, that live exclusively on phytoplankton – and predators, organisms, that feed on consumers and predators.

The cycle of elements is determined chiefly by the uptake from and release into the milieu of chemical components by the living organisms, together with the abiotic processes in the surrounding medium which are indispensable to convert the excreted compounds into substances, that can be absorbed again. The cycle consists of a great number of continuously interlocking conversions. It is, however, useful to divide the cycle artificially into a number of discontinuous steps; an element is considered to have changed from one step to the next, when it has been transferred to a compound, classed differently in chemistry. The requirements for this classification can be different for various cases. Ecological equivalence is more important here than chemical equivalence. When a thus defined transfer has resulted from a change in the cycle of organisms, we speak of a link between both cycles.

A link between both cycles is met in photosynthesis. Another link occurs, when an alga dies – by whichever cause – and returns to the water a part of the assimilated elements. In general the various elements are given off from dead algae into the water at different rates, some compounds having such a low rate of dissolution, that we are inclined to speak of slags. The type of the biocoenosis determines the nature and the amount of the slags. Small modifications in the

composition of the biocoenosis can involve the conversion of a slag into an absorbable compound; this can also be effected by abiotic processes.

Phenomena, in principle analogous to those happening at the death of an alga, occur at its consumption by a heterotrophic organism, or, more generally, at the transfer of matter from one organism to another. For, when a consumer contains two elements in a ratio differing from that in the consumed organism, the quantity of the element, present in the latter in excess, will be excreted by the former and therefore liberated. The other element will be liberated later, viz. when the consumer in turn dies off. Here also the originally simultaneously bound elements reappear in the water at different moments.

Caused by these processes shiftings in the mutual ratios of the concentrations occur. These are often of greater importance than the concentrations of certain chemical substances as such for the selection of the organisms, which, under certain outward circumstances, get a chance to develop.

Thus, for instance, we may observe that the consumption of organism A by B opens up the way for C to develop, either because A feeds on C, or because A through its composition disturbs the concentration ratio, necessary for the growth of C, and this ratio is restored after the consumption of A by B. In the latter case the influence comes about via the cycle of elements. This cycle is as much dependent on as it is necessary for the cycle of organisms, as least as long as we can exclude external influences. For, the concentrations of the chemical elements, necessary for growth, and the forms in which those elements occur, are dependent immediately on the passage of these elements through the various organisms. Nitrogen, which may occur in the water in various organically bound forms, as three different ions and finally even as dissolved N_2, is an excellent example of this. It is forthwith clear, that we have a very complex situation here, about which very little is known quantitatively.

The possibilities here mentioned are only a few out of many, but they serve to illustrate that the starting-point for any ecological-hydrobiological research must be the knowledge – both analytical-chemical and physiological – of the individual needs and composition of the various organisms involved. Composition and structure are important because the nature of the involved material determines whether in a change of circumstances the elements will find their way through the cycle easily or with difficulty.

Understanding of the whole of processes enacted in a lake can only be acquired by approximation of the separate facets, by dividing the biocoenosis and through it the cycles in small parts and subjecting these isolated parts to a quantitative investigation in a laboratory. After that it must be tried to fit the acquired data into a larger ecological unit, where every change in a concentration results from the difference between increase and decrease, out of which at least one process has to be known quantitatively to acquire an idea of what

really happens. The fitting of data from a smaller into a larger ecological scale will have to be done by degrees, by changing from the laboratory to big tanks of several cubic meters, filled with known starting-material, and subsequently to an investigation in a natural lake.

The first requirement to be made for quantitative laboratory investigation is the determination of the distribution of a chosen element over the various ecologically determined fractions, while the sum of these fractions always has to be equal to what was present at the start of the experiment. The greatest difficulty is here that it has to be possible to draw a sample at random from the total amount of material present in the experiment. It must be feasible to make the studied object homogeneous.

To obtain a square balance of the material we have set ourselves the restriction for the present to involve no elements in our investigation that can take a part in the processes in gaseous state. Otherwise the work would have to be done in a fully closed system, which entails experimental difficulties.

The facet dealt with in this paper – which is to be considered, not only as a survey of the results obtained, but even more as a working-programme – concerns the passage through algae of certain elements, which are essential for the growth of these algae. We have restricted ourselves for the present to nitrogen, phosphorus, silicium and iron.

More than to the uptake of these elements by the algae – to which moreover rather much attention has been paid in literature – we have directed our attention to the question what the fate of these elements is, when an alga dies. Does the dead cell withdraw the absorbed elements from the cycle of elements for a prolonged time, thus rendering impossible a subsequent bloom of algae, or do these elements leave the cells speedily by whatever mechanism? It is of great importance here to be acquainted with the differences in dissolution rates of the various elements and the ratio between the amount of incorporated material and the amount of still present reserve material.

The solution of this problem in ecology is of great importance for the question, what the influence is on the cycle of the in nature often occurring bloom of algae – the curious phenomenon that, usually in spring and autumn, a special alga develops suddenly in quantity in a certain lake and often dies off at an even quicker rate.

We have been able to answer this question in part, by which it has become possible in principle to foretell, what changes will occur in a lake, when a once appeared bloom of algae dies off again. When moreover the most favorable conditions for the growth of the more commonly occurring algae are known, it must be possible to foretell more or less, what will happen in the next period.

When this work will be extended to other organisms or to an investigation in lakes, it will become more and more difficult to meet the above made experimental requirements, especially when the

biocoenosis deals with organisms of a larger size and also when the organisms can cause an inhomogeneous distribution over the milieu. To a lesser extent we encounter this difficulty also in nature when working with algae, because they accumulate in the upper, more illuminated, layers and are deposited in the lower layers after dying off. In the laboratory it was always possible to make homogeneous suspensions, by stirring through aeration or by shaking.

It is outside the scope of this work to go extensively into the function of the mud in a lake and the experimental difficulties which may arise there. The determination only of the concentration of an element in water in contact with mud is quite senseless, when one can not at the same time determine the possibly important reserve stock of that element in the mud. Notwithstanding repeated efforts – partly with the use of radio-active isotopes – there is as yet no method known to measure satisfactorily the buffering effect of mud on the cycle of elements.

In laboratory experiments we can make an instantaneous picture at any moment of the distribution of an element over its compounds and of its localisations; in investigations in natural water this will hardly ever be the case. But it may prove to be of significance, when we can give meaning to a certain phenomenon in a lake on account of similar observations obtained from laboratory experiments.

CHAPTER II

THE ORGANISMS

Choice of object

As has been mentioned in the introduction, we wanted for our investigation an alga that is a common inhabitant of the dutch lake water, and that can be easily cultivated under conditions similar to those in a lake, especially as far as the concentrations of the required nutrients are concerned, while its chief physiological properties must be rather accurately known.

Of the algae obtained by us – vid. page 6 – *Scenedesmus quadricauda* and *Stephanodiscus Hantzschii* meet these requirements very well and were used in our experiments.

Technique of isolation

Bacteria-free, unialgal cultures were obtained in different ways.

Lake water was placed in the laboratory in the light till a growth of algae became distinctly visible. When necessary 70 mg of KNO_3 and 5 mg of KH_2PO_4 were added to 1 liter of water, the concentrations being nearly equal to Rhode's culture medium No. 8 (Rhode 1948). The algae were isolated from this solution, either with a micropipette or by inoculating in Petri dishes on an agar-medium, consisting of enriched lake water solidified by 2.5 % agar (Pringsheim 1946). The first method is not suitable for diatoms, because these may become attached to the glass.

Sterility was tested by means of peptone-glucose plates or of liquid media. Not always the obtained cultures proved to be sterile. Repeated subculturing of the non-sterile cultures on Petri dishes usually led to sterile cultures. Occasionally penicillin was added.

The following species were obtained:

Scenedesmus quadricauda (Turpin) Kützing
Ankistrodesmus falcatus (Corda) Ralfs
Nitzschia communis Rabenhorst
Chlamydomonas, not further identified
Ankistrodesmus convolutus Corda
Gonium pectorale O.F.M.
Stephanodiscus Hantzschii Grun.
Chlorella vulgaris Beyerinck
Stichococcus bacillaris Naegeli

The bacteria-free cultures were kept, partly in liquid media, partly in agar media in tubes. The stock cultures were subcultured every two or three months.

Culturing

As a culture medium for the green algae we used Rodhe's culture solution No. 8 with a few minor modifications. The amount of $Ca(NO_3)_2$ used was only 10 % of that indicated by Rodhe. As a source of nitrogen we used NH_4NO_3, the pH of the solution remaining more constant then during the growth of the algae. $NaHCO_3$ was added, when a rapid growth was required. Fe-citrate was substituted by Fe-EDTA (ethylenediaminetetraacetic acid), because this stock-solution can be kept longer. In a few special experiments, in which no nitrate was allowed to be present, NH_4Cl was used, whereas $Ca(NO_3)_2$ was substituted by $CaCO_3$, which counteracts a possible acidification of the medium. More EDTA is then added, because otherwise Ca binds the EDTA of the Fe-EDTA solution.

Modified Rodhe's culture medium for green algae:

$Ca(NO_3)_2.4\ H_2O$	10 mg	or $CaCO_3$	50 mg + EDTA		185 mg;
$MgSO_4.7\ H_2O$	10 mg;	KH_2PO_4	5 mg;	NH_4NO_3	30 mg;
$NaHCO_3$	75–100 mg;	Fe-EDTA sol.[1])	5 ml;	H_2O	1000 ml.

The green algae could be cultured on this medium for long periods. Some of the species we have been subculturing for six years now.

The diatoms could not be cultured on this medium, when only Na_2SiO_3 was added. They grew fairly rapidly after adding purified yeast-autolysate, or according to Pringsheim (1946), soil-extract. In high concentrations non-purified yeast-autolysate proved to act as an inhibitor.

Modified Rodhe's culture medium for diatoms:

$Ca(NO_3)_2.4\ H_2O$ 10 mg; $MgSO_4.7\ H_2O$ 10 mg; $Na_2SiO_3.9\ H_2O$ 50 mg; KH_2PO_4 5 mg; NH_4NO_3 30 mg; Fe-EDTA-sol.[1]) 5 ml; H_2O 1000 ml, Soil extract 20–50 ml; purified yeast-autolysate 5–10 ml.

The purified yeast-autolysate was prepared as follows:

1 Kg of yeast in 1 liter of H_2O is kept at 60° C during one night. This suspension

[1]) In a solution of 0.8 g of Na_2-EDTA per liter 1.0 g of $(NH_4)_2SO_4.Fe_2(SO_4)_3.24\ H_2O$ is dissolved without heating.

is boiled and after cooling centrifuged. To the supernatant three times its volume of acetone is added, followed after one hour by filtration. The filtrate is evaporated to 200 ml and kept in a refrigerator for one night. After filtration a clear pale yellow solution is obtained, which, after sterilisation, can be stored for many months. Higher concentrations than the above mentioned had neither a more stimulating nor an inhibiting effect on the growth of the diatoms.

Instead of by yeast-autolysate or soil-extract, modified Rodhe's No. 8 solution could be improved also by using p.a. chemicals (Merck or BDH) and adding $NaHCO_3$ and $CaCO_3$. As no Na_2SiO_3 p.a. is obtainable, this was purified by precipitation of the SiO_2 with H_2SO_4, washing with water and fusing afterwards with $NaHCO_3$ p.a. at 1000° C.

With p.a. chemicals and purified Na_2SiO_3 the growth of the diatoms was as good as in modified Rodhe's solution with purified yeast-autolysate or soil-extract; the only difference being, that they deteriorated quickly in the light after the growth had stopped by lack of nutrients.

The cells were cultured in Fernbach-flasks or in flat-bottomed 10 liter flasks.

The Fernbach flasks are filled with one liter of culture medium and closed with rubber stoppers provided with an inlet and outlet of air; the latter is also used for inoculating. Seven of these flasks are placed in an aquarium tank of 3 × 15 dm with a glass bottom, under which two fluorescent lamps (Philips 65 Watt) have been mounted, so that the distance to the flasks is about 15 cm. Through the tank – filled with distilled water – run two metal tubes, through which tap-water can be led, via a waterseal operated by means of a thermostate. As a rule we cultured our material at 20° C.

For special purposes the cells are also cultured in 10 liter flat-bottomed flasks. Through the rubber stoppers are now fitted three tubes, the inlet and outlet of air and a siphon. The inlet ends 5 cm above the bottom of the flask in a horizontally placed quarter circle with a radius slightly smaller than that of the flask at that level. In this glass loop holes have been made of about 1 mm diameter, spaced at a distance of 1 cm, causing sufficient bubbling of air to keep the green algae in a homogeneous suspension, which is not the case in the aeration of the Fernbach flasks. The inlet is, via a rubber tube lined inside with talcum-powder, connected with a small bell-jar closed with cotton-wool. The rubber tube is closed during sterilisation. Inoculation is performed through the air outlet. Samples of the culture medium can be siphoned out of the flasks without contamination, as the outside leg terminates in a little bell-jar with a downwards facing aperture. During sterilisation the siphon is drawn out of the liquid and closed with cotton-wool and afterwards pushed down and closed with a rubber stopper.

This apparatus enables us to obtain about 8 liters of culture, from which at different moments about 5 liters in total can be drawn and, if required, replaced by fresh culture medium.

Most experiments were carried out with *Scenedesmus quadricauda*. We also cultured *Stephanodiscus Hantzschii* and *Nitschia palea*, both of which

grew better without aeration. A disadvantage was, that the diatoms clot together very closely. A homogeneous suspension was best obtained by carefully shaking the diatoms with glass pearls of 5 mm and removing the remaining clots by filtering over glass wool.

At two to thee weeks' intervals so much nitrogen, phosphorus, silicium, iron and magnesium was added to the remaining cultures, that the original concentrations were attained once more. We noticed, that after a few days the culture solution contained no traceable amounts of silicium and phosphorus any more. For nitrogen the same applies after a slightly longer period. After depletion of nitrogen and phosphorus the dry weight of the cells can still increase for a prolonged time.

Filtration and centrifugation

The quantitative separation of *Scenedesmus* cells and their liquid medium can be obtained in principle by filtration or centrifugation. Both methods have their specific difficulties.

For filtrations very hard filters have to be used, entailing very low filtration-rates. The filters are soon blocked, thus rendering subsequent washing of the cells impossible. It is also very difficult – usually even impossible – to remove the cells from the paper. This method is therefore only applicable when the filtrate is wanted. When the cells have to be isolated, the suspension has to be centrifuged. The best method is to concentrate the suspension by leaving the cells to settle, and to centrifuge afterwards, as this decreases the disturbing whirling of the cells. In this connection it is important to remark that the centrifugation tubes with round bottoms give the best results.

Repeated washing of an amount of *Scenedesmus* cells entails increasingly worse results.

Recently we obtained good results by using Schott G 5 M glass filters. This method proved to be successful for obtaining cells (even washed ones) as well as fluid.

In the course of our experiments we had to centrifuge the *Scenedesmus* cells from solutions of 20 % and 60 % ethanol and 5 % trichloroacetic acid. No difficulties were met with, even after repeated washings. This did not apply to *Ankistrodesmus* and the diatoms used by us. The separation between fluid and these cells was effected by filtration through a Schott G 5 M glass filter.

CHAPTER III

METHODS

In this chapter a survey is given of the various analytical methods used. In general the common procedures were applied; the modifications that were necessary are discussed in detail. Each procedure used was tested thoroughly for recovery of known quantities added.

It has to be stressed that any given procedure yielding satisfactory results for our extracts and the lake water used by us is not necessarily suited to any other type of water.

All determinations were carried out colorimetrically by means of the Zeiss photometer Elko III.

Determinations

Ammonia

The amount of ammonia, present in the solution (lake water or culture medium) was determined by distillation of the alkaline solution and nesslerization of the distillate.

An aliquot of the solution, containing less than 1 mg of $N(NH_3)$, is made alkaline with a saturated borax-solution with phenolphtalein as an indicator, to pink colour, and distilled with a microdistillation apparatus after Parnas–Wagner into a 30 ml volumetric flask, containing 2.5 ml of 0.03 N H_2SO_4.

It is imperative to have the aperture of the condenser end under the surface of the H_2SO_4. If this is omitted – as happens rather frequently – losses up to 30 % may occur.

1 ml of Nessler's reagent is added to 20 ml of distillate. The extinction is measured, after one hour at room temperature, by means of filter 42. If necessary, the distillate is diluted 4 times beforehand. The extinction is proportional to the quantity of $N(NH_3)$ up to 200 μg. The smallest quantity that can be determined with an experimental error of 1.5 % is 3 μg when a 5 cm cuvette is used.

Nessler's reagent is prepared by dissolving 100 g of HgI_2 (red) with 70 g of KI in 400 ml of H_2O. After adding 100 g of NaOH in 500 ml of H_2O, the solution is diluted to 1 liter (Snell and Snell 1954). This reagent can be stored in a refrigerator for several months.

This method of preparing Nessler's reagent is more satisfactory than that, generally used, in which $HgCl_2$ is dissolved in an excess of KI, in which case the calibration curve is often not a straight line and the molecular extinction varies greatly.

Nitrite

An aliquot of the solution to be tested on NO_2', containing less than 35 μg of $N(NO_2')$, is diluted to 100 ml in a 100/110 ml volumetric flask. At room temperature are added 5 ml of a 0.2 % sulphanilamide solution in H_2O, immediately followed (or simultaneously) by 2 ml of a 20 % HCl solution, after 3 minutes by 1 ml of a 0.5 % ammonium-sulphamate solution in H_2O, and after another 3 minutes by 1 ml of an 0.1 % solution of N-(1-naphtyl)ethylene diamine.di-HCl in H_2O. (This reagent has to be kept in a refrigerator).

Finally the solution is diluted to 110 ml.

The extinction can be measured after 15 minutes by means of filter 53 and is proportional to the quantity of $N(NO_2')$ up to 35 μg. The smallest quantity that can be determined with an experimental error of about 1.5 % is 1 μg of $N(NO_2')$ per 100 ml of the solution in a 5 cm cuvette. The blank is prepared by addition first of the ammoniumsulphamate solution and then of the other reagents.

This method is similar to that described by Shinn (1941) and

modified by SIJDERIUS (1954). Sijderius, however, adds first the HCl and then the sulphanilamide. In the lake water in question we recovered by this procedure only 90 % of the nitrite added. This deficit increased, when the interval between the addition of the HCl and the sulphanilamide is prolonged. It is obvious, that the HNO_2 disappears from the acidified lake water.

Nitrate

Nitrate was determined by means of the xylenol method as published elsewhere (GOLTERMAN 1955).

The extinction is proportional to the quantity of $N(NO_3')$ up to 500 μg. The smallest quantity that can be determined with an experimental error of 1.5 % is 1 μg of $N(NO_3')$ (5 cm cuvette).

Total nitrogen

The determination of the soluble organically bound nitrogen (N^{sol}_{bound}) and the in algal cells incorporated nitrogen (N^{cell}_{tot}) was carried out by means of digestion after Kjeldahl.

An aliquot of the material to be analysed is heated with 2 ml of H_2SO_4 96 % and 10 drops of a 10 % $CuSO_4$ solution in H_2O till the digest has been colourless for at least 15 minutes. When much carbon appears during the destruction, the product is left to cool after complete carbonisation, and 10–20 drops of a 30 % solution of H_2O_2 are added to it, after which it is heated again until it is colourless. In the digest the NH_3 is determined in the usual way; for the alkalinisation a 10 % NaOH solution is used.

The difference between the total amount of nitrogen in the solution (N^{sol}_{tot}) and the amount of $N(NH_3)$ is the quantity of the soluble bound $N(N^{sol}_{bound})$; the difference between the amount of nitrogen in a suspension (N^{susp}_{tot}) and in its filtrate (N^{sol}_{tot}) is the total quantity in the cells (N^{cell}_{tot}).

Inorganic phosphate

Originally the inorganic phosphate (PO_4''') was determined by measuring the extinction of the blue colour of phosphomolybdate, reduced with $SnCl_2$, in 1.0 N H_2SO_4. However, as we wanted to determine phosphate in the presence of labile phosphate-esters, and also in digests with a varying amount of H_2SO_4, this method was not satisfactory. Good results were obtained by the procedure after Berenblum and Chain, as modified by MARTIN and DOTY (1949). They extracted the phosphomolybdate formed with an isobutylalcoholbenzene mixture, after which an aliquot of the product was diluted with ethanol and subsequently reduced with $SnCl_2$.

Instead of extracting with a mixture of isobutylalcohol and benzene we used a mixture of n-butanol and benzene, as this appeared to separate quicker after shaking. We considered it simpler and more reliable to reduce the whole quantity of the extract. Moreover this makes the method more sensitive.

It is indispensable to determine the most favorable n-butanol-

benzene ratio. A mixture of about 80 % n-butanol and 20 % benzene (v/v) gave the best results (Exp. I). The extraction time was 15 seconds; longer periods did not yield a higher extinction, whatever ratio between n-butanol and benzene was chosen.

EXP. I. *The effect of the n-butanol-benzene ratio in the extraction solution on the* PO_4'''-*determination.*

6 ml of a 5 % ammoniummolybdate solution in 2.5 N H_2SO_4 was added to 25 ml of H_2O, containing 60 μg of P (PO_4'''). The solution was extracted during 15 seconds with 25 ml of a mixture of n-butanol and benzene. The yellow upper layer was washed carefully with H_2O, without shaking, and shaken during 1 minute with 6 ml of an $SnCl_2$ solution (0.5 g of $SnCl_2$ and 2 g of hydrazine-sulphate dissolved in 1 liter of 0.6 N H_2SO_4). The extinction of the blue upper layer was determined, after dilution with ethanol to a suitable known volume.

extraction-medium n-butanol	benzene	extinction × 10^3			
100 %	0 %	250			
50	50	275			
75	25	284			
77.5	22.5	307			
79	21	305	308		
80	20	306	301	308	308
81	19	305	308		
82.5	17.5	299			
85	15	298			
89	11	290			

The extinction did not vary, when during the procedure 3 to 10 ml instead of 6 ml of ammoniummolybdate reagent was added, neither when 5 to 10 ml of $SnCl_2$ solution was used. The maximal value of the extinction could only be obtained by shaking the solution during at least one minute with the $SnCl_2$ reagent. The yellow butanol-benzene layer could also be reduced in a homogeneous phase with a solution of $SnCl_2$ in ethanol or by diluting the mixture with ethanol and adding one drop of a 40 % solution of $SnCl_2$ in fuming HCl. Under these conditions the non-converted molybdate must be removed very carefully, which is possible but laborious.

The requirement was made above, that the method should also be suitable for both Kjeldahl-digests and eluates of ion-exchangers, containing varying amounts of H_2SO_4 and Na_2SO_4. Therefore the influence of both compounds was tested (exp. IIa and IIb).

These experiments demonstrate, that an amount of H_2SO_4 up to 28.5 maeq. in 30 ml of the reaction solution had no disturbing effect on the PO_4'''-determination. The same conclusion is valid for the effect of adding 2 g of Na_2SO_4, provided the reaction mixture is extracted repeatedly by the n-butanol/benzene solution.

Besides its relative independence of the H_2SO_4 or Na_2SO_4 concen-

EXP. IIa. *The influence of varying amounts of* H_2SO_4 *on the* PO_4'''*-determination.*

Experimental data, see Exp. I. 5 ml of a 5 % ammoniummolybdate solution in H_2O was used and varying amounts of H_2SO_4 were added.

μg P (PO_4''')	H_2SO_4 present in the reaction medium	extinction × 10^3/100 μg P			
50	12.5 maeq.	516	520		
	17.8	515			
	28.5	513			
100	12.5 maeq.	510	516	513	515
	17.8	516			
	20.5	520			
	28.5	514			
	35	450			

EXP. IIb. *The influence of varying amounts of* Na_2SO_4 *on the* PO_4'''*-determination.*

Experimental data: See Exp. I. — 5 ml of a 5 % ammoniummolybdate solution in 2.5 N H_2SO_4 was used as reagent. — Varying amounts of Na_2SO_4 were added.

μg P (PO_4''')	Na_2SO_4 added	Extinction × 10^3/100 μg P: after 1 extraction with 25 ml 80 % butanol/20 % benzene				after 2 extractions with 25 ml 80 % butanol/20 % benzene	
50	0.0 g	516	520				
	1.2	513					
100	0.0	510	516	513	515		
	1.2	490				515	
	2.0	480				508	516

tration of the reaction mixture, this method has the advantage, that small quantities of PO_4''', in relatively low concentrations, can be determined, if the solution is repeatedly shaken with the n-butanol-benzene mixture. In this way 2 μg of P(PO_4''') could be determined in 250 ml of H_2O with an error of 2 %.

As the H_2SO_4 needs to be present in the reaction medium only for a very short time (less than one minute), the inorganic phosphate can be determined beside labile phosphate-esters. (LINDBERG and ERNSTER 1956).

The procedure for the PO_4'''-determination runs as follows:

To 10 to 35 ml of PO_4'''-solution is added 5 ml of a 5 % solution of ammoniummolybdate in 2.5 N H_2SO_4, if the solution does not contain more than about 14 maeq. of H_2SO_4, and 5 ml of a 5 % solution of ammoniummolybdate in H_2O if the solution contains 14 to 28 maeq. of H_2SO_4. 12 to 15 ml of a mixture of n-butanol and benzene (8 : 2,

v/v) are added. After shaking for 15 seconds and complete separation of both layers the lower layer is removed. Some water is squirted inside the separatory funnel and let out again without shaking, by which the molybdate reagent, still adhering to the glass, is removed. The phosphomolybdate-solution is then shaken during one minute with a reducing agent, which is prepared by dissolving 2 g of hydrazine-sulphate through heating in 1 liter of 0.6 N H_2SO_4 and adding, after cooling to about 15° C, 0.5 g of $SnCl_2$ (this solution can be stored in a refrigerator for months).

The lower layer is removed completely and the upper layer diluted to a suitable known volume with ethanol 96 %. The extinction is determined with filter 72.

It is advisable to make the separatory funnel water-repellent with Desicote and to use a silicone-grease for the cocks.

The amount of phosphate, that can be determined in this way, is 1 to 200 μg of $P(PO_4''')$. The calibration curve is straight in this area. the experimental error is about 1.5 %.

Finally we draw attention to a disturbance, which can occur in some kinds of water or of cell-suspensions, when the aqueous lower layer also colours blue after reduction. This could be avoided by adding to the sample 20 maeq. of H_2SO_4 and so many drops of 0.1 N $KMnO_4$ that, after being left standing for 15 minutes, the last 2 or 3 drops did not lose colour. Subsequently the mixture of n-butanol and benzene is added and the two layers are shaken together for a moment, by which the excess of $KMnO_4$ is removed. After this the solution of ammoniummolybdate in H_2O is added and the determination carried out as described.

Total phosphate

The material, in which originally bound phosphate occurs, is destructed in the usual way with 2 ml of H_2SO_4 96 %. The digestions were carried out in Kjeldahlflasks of 25 to 50 ml with a neck of about 30 cm to counteract the evaporation of P_2O_5 with the H_2SO_4. The neck serves as a reflux-condenser.

After cooling the solution is boiled with about 10 ml of H_2O and kept hot for 10 minutes to bring about decomposition of the possibly formed pyrophosphates, after which the solution is cooled and diluted to 30 ml. 5 or 10 ml of this product, containing resp. 12 and 24 maeq. of H_2SO_4, can be used directly for a PO_4'''-determination with an ammoniummolybdate solution in H_2O.

When H_2O_2 has been used as a catalyst for the digestion, the digest is treated twice with water. In the first treatment it is distilled off to remove the H_2O_2 that had not been decomposed and in the second one the pyrophosphate is decomposed.

The difference between the total amount of phosphate in the solution to be tested (P^{sol}_{tot}) and the amount of PO_4''' is the quantity of the soluble bound P (P^{sol}_{bound}); the difference between the amount of phosphate in a suspension (P^{susp}_{tot}) and its filtrate (P^{sol}_{tot}) is the total quantity in the cells (P^{cell}_{tot}).

Silicate

SiO_3'' was determined according to the method of Milton (1951). It is converted by Na-molybdate to silicomolybdate, which is reduced with $SnCl_2$ to a blue coloured compound.

Milton recommends to form the silicomolybdate complex at 100° C, as in his experiments this reaction was not complete at 20° C and higher temperatures lead to higher extinction values. We found, however, that at 20° C the reaction was complete and resulted in a constant extinction value, although a lower value was obtained than at 100° C. Contrary to Miltons findings heating has to our experience the disadvantage of colouring the blank far more intensively. For these reasons we carried the reaction out at room temperature, as the method was moreover sufficiently sensitive for our investigations.

The determination is carried out as follows:

An aliquot of the liquid to be tested (pH 5–8), containing 1 to 200 μg of Si(SiO_3'') is diluted with water to 15 ml. 2 ml of a molybdate solution is added, containing 5 g of $Na_2MoO_4 . 2H_2O$ in 100 ml of 0.5 N H_2SO_4. This reagent can not be used sooner than 48 hours after it has been prepared, as this would lead to varying extinction values.

15 minutes later 2 ml of H_2SO_4 (s.g. 1.84) is added and the solution cooled to room temperature. Finally 1 ml of a 1 : 100 with water diluted $SnCl_2$ solution is added in drops. The stock solution contains 40 g of $SnCl_2$ dissolved in and diluted to 100 ml with fuming HCl, and can be stored in a refrigerator for months.

The extinction of the reduction product, constant after 10 minutes, is measured with filter 75. Its value is proportional to the quantity of Si(SiO_3'') up to 200 μg. The smallest quantity, that can be determined with an experimental error of 1.5 % is 1 μg (5 cm cuvette).

As a standard we used $Na_2SiO_3 . 9\ H_2O$, which was 99.7 % pure according to acidimetric and gravimetric (crystal-water content) determinations.

The SiO_3''-determination may be disturbed by the phosphate, present in the solution. This was rectified by determining the extinction of phosphate-standards, treated according to the procedure for SiO_3''-determinations, and correcting the obtained extinctions of the tested samples in this respect. It appeared, that this correction depends on the $Na_2MoO_4 . 2\ H_2O$ used, possibly through the presence of varying amounts of $Mo_7O_{24}^{6-}$. The cause of these differences has not been traced further; it is very probable, that the differences in Milton's and our findings can also be attributed to the Na-molybdate, although perhaps the difference between his and our standards is of importance too.

Total silicium

For the determination of the total amount of Si present in cells, a known quantity of the diatom suspension is evaporated till dryness with 2.50 g of $NaHCO_3$ in a nickel crucible and heated subsequently

in an electric furnace to 1000° C. After cooling the contents are dissolved in H_2O, neutralized with HCl and diluted with H_2O to a known volume (100–1000 ml), depending on the expected Si-content. Then Si^{susp}_{tot} is determined with the method described above.

The value of Si^{cell}_{tot} is calculated as the difference of Si^{susp}_{tot} and SiO_3''.

Fractionation of the compounds in Scenedesmus quadricauda

As it is not feasible, nor necessary, to carry out a complete analysis of the conversions of all compounds involved in the chemical cycle of algal cells, the investigation was restricted to a division into groups of compounds. Then it can be followed up, how the ecologically equivalent compounds are distributed over these groups.

The following fractionation technique proved to be very useful in practice for P- and N-compounds:

The algal cells were boiled after adding ethanol to the suspension to a concentration of 60 %. The alcohol-soluble compounds (fraction alc 60 %) were separated by centrifugation from the insoluble residue (residue A), which was suspended at 4° C in a 5 % trichloroacetic acid (TCA)-solution. The fraction soluble in it (Fraction TCA-c) was separated from the second residue (residue B). Some of the compounds in this residue could be dissolved under partial hydrolysis either with a 5 % TCA-solution at 90° C (fraction TCA-w) or with 0.25 N KOH at room temperature. All extractions could be carried out in such a way that reproducible and sharply defined fractions were obtained; repetitions of each extraction did not result in an increase of the quantity of the compounds extracted.

Boiling of the cells with alcohol, to obtain a first fraction, has the additional advantage of stopping instantaneously all processes, occurring in the cells, either before or after their breakdown.

Table I (Page 19) demonstrates, that under different culturing conditions the relative amounts of the P-compounds in the different fractions may vary considerably. The same holds true for other compounds.

In the following sections is described more in detail which P-compounds are extracted in each of the fractions.

a. *Alcohol-soluble fraction*

This fraction is obtained by boiling the cells in alcohol of 60 to 65 % for 5 minutes and setting the suspension aside at room temperature for 1 to 2 hours, to give the compounds time to leave the cells (exp. IIIb). It makes no difference whether the cells are left at 0° C or at 25° C.

The cells are centrifuged off and in the supernatant the amount of P-compounds, P^{cell}_{alc}, is determined. This amount does not depend on the alcohol percentage as long as this remains between 60 and 70; outside this range it diminishes quickly.

Boiling of the cell suspensions for a time as short as 5–15 minutes does not cause a noticeable hydrolysis of insoluble to soluble material, the P-content of the extract remaining constant. Over longer boiling-

periods the yield does increase and reaches a second level (Exp. IIIa). The extra amount of phosphate, dissolved in the alcohol after a 24 hours' boiling period (in exp. IIIa 43 % — 20 % = 23 %) is formed through hydrolysis out of phosphate from the fraction TCA-w. In accordance with this the fraction TCA-c also increases, viz. from 16 % after 5 minutes to 45 % after 24 hours boiling. Owing to this hydrolysis the obtained extracts are not suitable for further analysis.

Attempts were made to avoid the heating of the suspension by disintegrating the cells mechanically either in an ultrasonic homogenizer or in a blendor at 50000 r.p.m. or in a mortar with quartz or carborundum. These experiments yielded no results, as only part of the cells were damaged.

EXP. IIIa. *Influence of boiling time on the amount of* P-*compounds extracted with alcohol* 60 %.

To cell suspensions, which did not contain PO_4''' any more, ethanol was added to a concentration of 60 %. In an aliquot P_{tot}^{susp} was determined. Samples of these alcoholic suspensions were boiled in a waterbath under a reflux condenser during different periods, after which they were cooled off quickly to 20° C. 24 hours later the samples were centrifuged and the P-content of the supernatant, P_{tot}^{alc}, was determined.

Boiling period	P_{tot}^{alc} in % of P_{tot}^{susp} susp. a	susp. b
5 min	21.4	20.0
10 min	21.0	20.0
15 min	21.5	21.0
20 min	—	21.0
25 min	23.6	—
180 min	40.2	—
6 hrs	48.5	39.0
20 hrs	—	43.0
24 hrs	—	43.0

EXP. IIIb. *Influence of the extraction time on the amount of* P_{alc}^{cell}.

In this experiment the alcoholic cell suspension was heated for 15 minutes. The extraction period at 20° C varied from 0.5 to 24 hours. Other data as in experiment IIIa.

Extraction time	P_{alc}^{cell} in % of P_{tot}^{cell} susp. c	susp. d
0.5 hr	18 20	—
1 hr	20	—
2 hrs	20	23.7
4 hrs	—	24.2
6 hrs	—	23.7
24 hrs	—	23.8

Some of the P-compounds of fraction alc 60 % – the phospholipids – can be separated from it by shaking out with an equal volume of $CHCl_3$, followed by a few hours' standing. They move quantitatively into the $CHCl_3$ after one extraction.

Addition of H_2O during this extraction is not allowed, for it appeared that $CHCl_3$, which can take up most of the alcohol – and with it the phospholipids and pigments – from an equal volume of a 60 % solution, can not do so from a larger volume of a 30 % solution.

In the $CHCl_3$-fraction total phosphate can be determined, when all $CHCl_3$ is removed very carefully by distilling and subsequently producing a vacuum in the still very hot Kjeldahl-flasks. The residue is digested with H_2SO_4 and $CuSO_4$ in the usual way.

The PO_4''' of fraction alc 60 %, which includes the PO_4''' originally present in the cells, can not be determined as long as alcohol or pigments are present. This determination is therefore carried out in the water-layer, remaining after the $CHCl_3$-extraction, which must be repeated twice after the extraction of the phospholipids to remove the last traces of alcohol. The organic P-compounds present in the water-layer can be determined as described above.

b. *Fraction, soluble in* TCA *at* 4° C

Residue A is suspended in a 5 % TCA solution at 4° C. Experiment IV shows, that a certain amount of P-compounds dissolves within 1 hour. As the concentration in the solution does not increase further, we may assume that a definite fraction of P-compounds has been extracted. The residue (residue B) is separated from the TCA-c extract by centrifugation.

EXP. IV. *Influence of the extraction time on the extraction with* TCA *at* 4° C.

Residue A was suspended in 50 ml of a 5 % TCA solution at 4° C. After 0.5, 1 and 2 hours samples were centrifuged, and the P-content of the supernatant, $P^{cell}_{TCA\text{-}c}$, was determined. P^{cell}_{tot} had been determined before.

Extraction time	$P^{cell}_{TCA\text{-}c}$ in % of P^{cell}_{tot} susp. a	susp. b
0.5 hr	—	11.0
1 hr	20.0	20.5
2 hr	21.0	20.8

Fraction TCA-c contains no PO_4''', from which follows that the PO_4''', originally present in the cells, has been extracted quantitatively with alcohol 60 %. It contains compounds, which will form PO_4''' through hydrolysis, when the temperature of the TCA-solution rises to room temperature.

The TCA-c extract shows a definite U.V. absorption spectrum with a maximum between 245 and 250 mμ when all the TCA has been previously removed.

At 4° C a 0.3 N $HClO_4$ solution extracts from residue A more compounds than a 5 % TCA-solution, as appeared from the U.V. absorption spectrum, although the quantity of P-compounds is equal.

A further fractionation of these extracts was not carried out, but might be done chromatographically or by precipitation with $BaCl_2$ at different pH's (ALBAUM 1950).

Increasing the TCA concentration to 10 % yielded no more P-compounds in this fraction. The polyphosphates, mentioned by WIAME (1949, 1958) can not be extracted quantitatively with a 5% TCA solution, from which can be concluded, that no polyphosphates are present in fraction TCA-c.

c. *Fraction soluble in* TCA *at* 90° C

From residue B a third fraction can be obtained by heating it with a 5 % TCA solution during 20 minutes at 90° C and subsequently leaving it to stand for 1 hour at 20° C.

Experiment v shows, that under these conditions amost all P-compounds of residue B are dissolved, ($P^{cell}_{TCA\text{-}w}$), under partial hydrolysis to PO_4'''.

EXP. V. *Influence of heating and extraction time on the extraction with* TCA *at* 90° C. Residue B was suspended in 50 ml of a 5 % TCA solution. In an aliquot P^{susp}_{tot} was determined. Samples were heated to 90° C for different periods. The suspensions were filtered after a 1 and 2 hours' standing at 20° C. In the filtrates P (PO_4''') and $P^{cell}_{TCA\text{-}w}$ were determined.

Suspension heated at 90° C for	$P^{cell}_{TCA\text{-}w}$ in % of P^{susp}_{tot}		P (PO_4''') in % of P^{susp}_{tot}	
	1 hr at 20° C	2 hrs at 20° C	1 hr at 20° C	2 hrs at 20° C
5 min	59	57	2	4
10 min	82	79	6	7
15 min	92	94	9	10
20 min	95	93	12	12

As is known, the TCA-w extract contains the nucleophosphates. Large quantities of P-compounds of other origin, called "polyphosphates" for the present, can also occur, as appeared from the fact, that different extracts had been obtained with the same extinctions, but a difference in P-contents of 500 %. As it could be expected that the nucleophosphates would react ecologically quite differently from the polyphosphates, it was important to determine the amounts of substances of both groups separately.

The quantity of nucleophosphate present was derived from the extinction values of the TCA-w extracts at 260 mμ. As, however, other compounds may occur in this extract with an absorption at the same wave-length (digestion products of proteins or plant pigments), it remains still necessary to find a more exact method for quantitative analysis of mixtures of nucleo-and polyphosphates.

CHAYEN (1955) has developed a method based on their different rates of hydrolysis to PO_4'''. This method, however, can not be used for our material, because in one experiment the nucleophosphate content determined after Chayen was about 4/5 of the value obtained by measuring the extinction at 260 mμ, while in another experiment it was about 2.7 times as high. In these two experiments the TCA was removed – together with the pigments – from the TCA-w extracts by shaking out twice with an equal volume of ether after addition of H_2SO_4 (0.1 aeq. per liter).

We discovered this method of removal after our series of experiments on autolysis had been closed.

We have not made use of the method of determination of polyphosphates by means of the metachromatic colour reaction with toluidineblue or of the precipitations with $BaCl_2$ at pH 2 and 4, because we were not sure that the phosphates, not belonging to the nucleophosphates, were indeed polyphosphates, and because we considered none of the methods, based on this supposition, sufficiently specific.

Table I demonstrates the varying ratios between the amounts of P-compounds present in the algal cells, depending on the species investigated and the culturing conditions.

TABLE I. *The distribution of the* P-*compounds over the fractions examined.*
The results are expressed as percentage of P^{cell}_{tot}. Data from experiments described in Chapter IV.

Exp.	Alga	P^{cell}_{alc}			$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$		P^{cell}_{rest}
3	Scen.q.	54%	—	—	15%	30%	—	—
5	id.	28	—	—	9	63	—	4
6	id.	27	—	—	11	56	—	3
7	id.	25	—	—	32	38	—	4
9	id.	25	—	—	12	54	(26) [3]	8
10	id.	33	—	—	13	57	(24) [3]	0
16	id.	9	—	—	5	82	—	2
17^{I}	id.	23	(12) [1]	+ (11) [2]	7	64	(32) [3]	3
17^{II}	id.	39	(19) [1]	+ (20) [2]	13	48	(18) [3]	0
17^{III}	id.	32	(16) [1]	+ (16) [2]	20	34	(10) [3]	7
18	Ankis.f.	16	—	—	2	79	—	3
19	Clado.g.	10	—	—	10	67	(23) [4]	13
20	id.	11	—	—	17	68	(45) [4]	4
21	Steph.H.	19	—	—	41	32	—	5
22	id.	23	—	—	28	42	—	7
23	id.	19	—	—	5	73	—	3

[1]) soluble in chloroform.
[2]) insoluble in chloroform.
[3]) P (PO_4''') after hydrolysis during 20 minutes.
[4]) P (PO_4''') after hydrolysis during 75 minutes.

CHAPTER IV

LIBERATION AND MINERALISATION

Introduction

The processes of liberation and mineralisation of elements play an important part in the chemical cycle in fresh water, when phytoplankton dies off. Ecologically, a distinction can be made between the decomposition processes through autolysis, effected only by the organism itself and through metabolic activity of other organisms, e.g. bacterial putrefaction. Consumption is, for the moment, left out of consideration.

The decomposition process starts with autolysis, which leads to an increase of permeability, enabling several compounds, already present in a dissolved state, to leave the cell. Moreover compounds, present in undissolved state, may dissolve. The temperature may be expected to exert a great influence, which can easily be determined, when the physico-chemical rate of the dissolution processes is the limiting factor. But it is also possible that the diffusion through the cellwall and protoplasm membranes limits the rates of dissolution and liberation. Here the size and nature of the molecule is of great importance. Nearly always these rates are increased by preceding hydrolysing processes. These conversions can either happen spontaneously or under influence of the cell enzymes present. Here, therefore, all kinds of factors, which can influence the enzymatic activity, may be of importance.

In order to study experimentally the part, that these autolysis processes have in the chemical cycle, it is necessary to kill the algae in a suspension completely, as quickly as possible and all at the same time, to prevent surviving cells from taking up liberated compounds from the dead cells. Furthermore the structure of the cells must be impaired as little as possible, because otherwise the diffusion rates of the various compounds may undergo inextricable changes. For this reason boiling of the cells in alcohol and other very vigourous means were rejected.

Three methods were used to kill the cells in a suspension: irradiation in an intensively shaken open glass dish (ø 25 cm) with U.V. light from three Philips T.U.V. lamps (30 Watt) mounted at a distance of about 10 cm for 0.5–4 hours; saturation with chloroform; heating during at least 2 hours at 60° C.

It appeared that the amounts of and the rates at which the P- and N-compounds leave the cells were equal when these three methods of killing were compared (Exp. 1, 3, 4 and 5). The only difference found was that the liberated P-compounds were hydrolysed to PO_4''' at a somewhat lower rate when U.V. light was used.

From this accordance can be concluded that the disturbing factors, mentioned above, were absent.

It might be considered whether some other poison than chloroform could be used. Mostly, however, lower yields were then obtained. When butanol, toluene or carbontetrachloride instead of chloroform were used, the same yields of extracted P-compounds were obtained eventually, but the starting rates of the decomposition processes were lower (Exp. 1–3). We did not investigate whether this was caused by a later penetration of the cells by these poisons, by a delayed dying off of the cells, or by an actually lower autolysis rate.

In principle U.V. irradiation is preferable, but, as the chloroform saturation prevents bacterial contamination, we usually made use of the latter method, as we carried out our experiments under sterile conditions. From one experiment it appeared that the autolysis continued normally, when the chloroform was removed after about one hour.

Liberation of P from Scenedesmus quadricauda

Table II gives an orientation about the rates of the different processes, which lead to the liberation of P-compounds. The cells were killed by chloroform treatment and the concentrations of the liberated P-compounds determined after different periods.

TABLE II. *Liberation of* P-*compounds from Scenedesmus at room temperature.*

The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period the liberated P (PO_4''') and P_{tot}^{water} were determined. For the experimental data see exp. 5 and 7, page 37 and 38.

Autolysis during	Experiment 5		Experiment 7	
	P (PO_4''')	P_{tot}^{water}	P (PO_4''')	P_{tot}^{water}
	in % of P_{tot}^{cell} at t = 0			
0 hrs	0 %	0 %	0 %	0 %
3.5 hrs	—	—	18	28
5 hrs	43	49	—	—
6 hrs	—	—	29	43
1 days	—	—	48	60
2 days	50	50	—	—
5 days	—	—	65	68
6 days	62	65	—	—
28 days	65	70	—	—

An important part of the P-compounds leaves the cells in a short time. In order to decide whether this is due to simple dissolution or to the action of hydrolysing factors, and whether the diffusion is limited or not, we investigated which P-compounds, grouped as described in Chapter III, contribute chiefly to this result.

a. *Liberation of the* P-*compounds of fraction alc* 60 %

Table III shows, that the P-compounds which can be extracted

from the cells with alcohol 60 %, consisting for about one half of phospholipids, have usually left the killed cells within 24 hours.

Never during or after autolysis can chloroform-soluble P-compounds be detected in the medium, so we concluded that the phospholipids are hydrolysed, probably to PO_4'''. Other cell compounds contributed too to the PO_4'''-liberation as the increase of PO_4''' exceeds the decrease of the phospholipids-phosphate. In the section on the influence of Mg^{++} and F' on the liberation (section e) is demonstrated that the hydrolysis comes about under influence of enzymatic activity.

TABLE III. *Liberation of* P-*compounds of fraction alc* 60 % *from Scenedesmus at room temperature.*

The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period P^{cell}_{alc} was determined. For the experimental data see exp. 4, 5, 6 and 7, page 35 etc.

Autolysis during (*days*)	P^{cell}_{alc} in % of P^{cell}_{tot} at t = 0			
	Exp. 4	Exp. 5	Exp. 6	Exp. 7
0	23 %	28 %	27 %	25 %
1	—	8	1	3
4	0	—	—	—
5	—	8	1	2

b. *Liberation of the* P-*compounds of fraction* TCA-c

Table IV shows, that the P-compounds in the cell, soluble in a 5 % TCA solution at 4° C after the extraction with alcohol 60 %, are liberated in the same short time (1–2 days) as the P-compounds of fraction alc 60 %.

The same compounds leave the cells in 1–2 days, when residue A – cells boiled in alcohol, in which therefore all enzymatic activity has stopped – is suspended in H_2O at 4° C or at 20° C. This proves, that the liberation of fraction TCA-c can take place without any enzymatic conversions.

The quantities, dissolved from residue A in H_2O after 1 day at 4° C, gave the impression, that the same liberation rate occurred, as is found in normally autolysing cells, which suggests, that indeed enzymatic processes do not play a role in the liberation of these compounds.

At 4° C these compounds dissolved nearly without hydrolysis to PO_4'''; at 20° C hydrolysis was considerable, which is quite in accordance with our findings that the P-compounds of fraction TCA-c are hydrolysed very rapidly to PO_4''' in a 5 % TCA solution at 20° C.

c. *Liberation of* P-*compounds of fraction* TCA-w

Some of the compounds in the cells, which can be dissolved in a 5 % TCA solution at 90° C after extraction with alcohol 60 % and

TABLE IV. *Liberation of* P-*compounds of fraction* TCA-c *from Scenedesmus at room temperature.*

The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period $P^{cell}_{TCA\text{-}c}$ was determined. For the experimental data see exp. 6, 7, and 8, page 38.

Autolysis during (*days*)	$P^{cell}_{TCA\text{-}c}$ in % of P^{cell}_{tot} at t = 0		
	exp. 6	exp. 7	exp. 8
0	11 %	32 %	10 %
1	—	2	7
2	3	—	—
5	—	2	7
12	—	2	—
14	—	—	4

with a 5 % TCA solution at 4° C, appear to leave these cells slowly after they are killed, while another part remains (Table v).

The liberation of the P-compounds of fraction TCA-w appears to be very variable. In experiment 7 the liberation took place very slowly, in some other experiments half of the fraction has left the cells in 4 days, while in experiment 6 only a small part is still present in the cells after 6 days.

TABLE V. *Liberation of* P-*compounds of fraction* TCA-w *from Scenedesmus at room temperature.*

The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period $P^{cell}_{TCA\text{-}w}$ was determined. In some experiments the PO_4''' content of the TCA-w extracts was determined (numbers between brackets). For the experimental data see exp. 4, 6, 7, 8, 9, 10 and 17^{II}, page 35 etc.

Autolysis during (*days*)	$P^{cell}_{TCA\text{-}w}$ in % of P^{cell}_{tot} at t = 0						
	exp. 4	exp. 6	exp. 7	exp. 8	exp. 9	exp. 10	exp. 17^{II}
0	42%	56%	38%	60%	54% (26%)	57% (24%)	48% (18%)
1	—	—	36	52	—	—	—
4	21	—	—	—	20 (4)	21 (4)	23 (7)
5	—	—	25	32	—	—	—
6	—	10	—	—	—	—	—
12	—	—	26	—	—	—	—
14	—	—	—	25	—	—	—

Various causes can be suggested, of which we mention here especially, that fraction TCA-w might consist of several compounds, present in varying amounts and liberated at unequal rates. An indication in this direction is found in the fact, that the amount of PO_4''' of this fraction (formed through hydrolysis from $P^{cell}_{TCA\text{-}w}$), fluctu-

ates between 35 % and 50 % before, and between 19 % and 30 % after autolysis.

In fraction TCA-w polyphosphates (WINTERMANS 1955, TAIICHI NIHEI 1955, 1957) and derivatives of nucleic acids can be present.

We have carried out some preliminary experiments to investigate whether the decrease of the amount of P-compounds in the fraction TCA-w during autolysis of the cells can be attributed to the nucleic acids diffusing out of the cells. For this purpose the extinction of the TCA-w extract at 260 mμ was determined before and during autolysis, the blank being a 5 % TCA solution which had been subjected to the same treatment.

We suppose that the decomposition of the TCA during the extractions is not influenced by compounds from the cells.

Apart from the extinctions, also $P^{cell}_{TCA\text{-}w}$ and P^{cell}_{tot} at t = 0 were determined (Table VI).

TABLE VI. *The fate of nucleic acids in Scenedesmus during autolysis at room temperature.*
The cells were killed by chloroform treatment and autolysed afterwards for different periods at room temperature. At the end of each period the extinction of the TCA extract at 260 mμ, $E^{cell}_{TCA\text{-}w}$, and $P^{cell}_{TCA\text{-}w}$ were determined.
For experimental data see exp. 8, 9, 10, 11 and 12.
mg of $P^{cell}_{TCA\text{-}w}$ per mg of P^{cell}_{tot} at t = 0.
$E^{cell}_{TCA\text{-}w}$ per mg of P^{cell}_{tot} at t = 0 per ml, and per mg of $P^{cell}_{TCA\text{-}w}$ per ml.

Autolysis during (*days*)	exp. 8			exp. 9			exp. 10		
	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$
0	0.60	71	120	0.54	52	96	0.57	52	91
1	0.52	71	137	—	—	—	—	—	—
4	—	—	—	0.20	51	255	0.21	52	250
5	0.32	71	222	—	—	—	—	—	—
14	0.25	71	284	—	—	—	—	—	—

Autolysis during (*days*)	exp. 11			exp. 12		
	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$	$\frac{P^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{tot}}$	$\frac{E^{cell}_{TCA\text{-}w}}{P^{cell}_{TCA\text{-}w}}$
0	0.38	74	195	0.42	100	238
3	0.185	44	240	0.21	59	280
4	—	—	—	—	—	—
7	—	—	—	0.21	—	—

It appeared, that in some experiments (8, 9 and 10) the quantity of nucleic acids did not change during autolysis. So we have to assume that the nucleic acids did not leave the cells. Somewhat divergent results were obtained with the experiments 11 and 12, both carried out with cells of the same culture.

It is possible in both latter experiments that the extinction at 260 mμ before

autolysis is caused not only by nucleic acids, but also by other compounds, and that those compounds leave the cells during autolysis. An indication, that this might happen, is found in the fact that in an experiment, not further mentioned, the extinction at 235 mμ decreased less than the extinction at 260 mμ which decreased by 20–30 %. For the determination of the extinction at 235 mμ the TCA present must be removed, which was achieved by extraction with ether.

That the disturbing compounds at 260 mμ do not occur in experiment 8, 9 and 10, may be a fortunate coincidence, dependent on the previous history of the cells.

Therefore it can not be excluded that in these experiments part of the nucleic acids liberated through unusual circumstances.

The values obtained in table VI for the extinction per mg $P^{cell}_{TCA\text{-}w}$ per ml after autolysis are in accordance with those, reported in literature, for the extinctions of nucleic acids per mg of nucleophosphate per ml. Thus CHAYEN (1955) gives 260–275 for native nucleic acid, WIAME (1949) 350, from data of DI CARLO et al. (1948) is calculated 275, BEAVEN et al. (1955) give 225–320. These statements vary, but Chayen has pointed out that the highest extinctions are obtained by working with commercial nucleic acid instead of native nucleic acid. By the alkaline extraction during the preparation of the commercial product a part of the phosphate is supposed to be split off.

Although it must be borne in mind, when comparing our own values with those cited, that the cited ones refer to yeast- instead of algal nucleic acid, we feel justified in concluding on the strength of the accordance between both groups of values, that the P-compounds remaining after autolysis consist of nucleophosphate.

We conclude from these preliminary experiments that the nucleic acids do not leave the cells during autolysis.

The values obtained in table VI for the extinction per mg $P^{cell}_{TCA\text{-}w}$ per ml before autolysis showm, that at that moment still other P-compounds, "polyphosphates", are present in the cells, also belonging to fraction TCA-w, but which do leave the cells. They are hydrolysed to PO_4''' more easily than the nucleic acids (Chayen). The method of quantitative analysis, based on this difference, did not yield good results for our material (Chapter III, page 19). It could be established, however, (Table V, exp. 9, 10 and 17^{II}) that the part of the P-compounds, hydrolysed during extraction with a 5 % TCA solution at 90° C to PO_4''', is smaller after autolysis than before.

d. *Liberation of fraction rest*-P

The P-compounds which can be extracted from the cells neither by ethanol 60 % nor by a 5 % TCA solution, at 4° C or at 90° C, are not liberated during autolysis. They constitute about 5 % of P^{cell}_{tot}. According to TAIICHI NIHEI (1957) these compounds are phosphoproteins. Their phosphate, like that of the nucleic acids, is only liberated by means of bacterial digestion, which is discussed in section 3b.

e. *Influence of external factors on the liberation of* P-*compounds*

In section c was shown, that variation in the nucleophosphate-polyphosphate ratio can be a cause of variability of the liberation of the P-compounds of fraction TCA-w.

It might also be considered, whether a variation in activity of the enzymatic systems, connected with it, could lead to similar effects, not only for fraction TCA-w, but for all fractions. To check this, the influence of the temperature, the pH and of Mg-, Ca- and F-ions on the liberation of P-compounds was determined.

Influence of temperature

As the dying off of algae in nature can occur at different temperatures, it is important to investigate the dependence of the liberation of P-compounds during autolysis on this factor.

In exp. 4, 5, 8 and 15 the liberation at different temperatures was determined. These experiments show, that during autolysis the liberation of P-compounds at 0° C does not differ significantly from that at 10° C. At higher temperatures both the amount of PO_4''' and that of P_{tot}^{water} increase, reaching a maximum at 30° C. At these high temperatures practically all the P liberated was present as PO_4'''.

From these data it can not be concluded on which of the mechanisms mentioned on page 20 the temperature exerts its influence. For this purpose the different fractions must be examined separately —preferably by short during experiments.

The temperature is an important ecological factor for the liberation of P-compounds in nature. It is of some importance to know, whether the liberation of P-compounds during autolysis at low temperatures finally equals that at higher temperatures, and if such is not the case, to know what happens, when the cells are brought from 0° C to 20° C after a long period. This was investigated in experiment 14. The liberation of P-compounds at different temperatures was compared. It appeared that in 12 days at 10° C about 2/3 was liberated of the amount of P-compounds liberated at 20° C. When the autolysis took place only a few days at 0° C and proceeded afterwards at 20° C finally the same amount of P-compounds was liberated as in the sample in which the cells remained constantly at 20° C. It appeared from this experiment that the enzymes connected with the liberation of P-compounds had not been damaged after a ten days' storage at 0° C. Longer periods have not yet been tried.

Influence of the pH

Several experiments were carried out, in which the influence of the pH on the liberation of P-compounds was investigated. In these experiments the pH was kept constant by means of an 1/20 M succinate-buffer (pH 3–6) or a 1/20 M tris-maleic acid buffer (pH 5–10). No differences were observed whether one of these buffer solutions had been added or not, when they had the same pH as the suspension medium, from which appears that they had no specific

effect. Between pH 5 and 9 the liberation does not depend on the pH, outside this range it decreases rapidly.

Influence of Ca^{++} *and* Mg^{++}

As it is probable that the phosphatases play a role in autolysis, we investigated whether Mg^{++} has a stimulating effect on the liberation of P-compounds. This could be expected as Mg^{++} is a constituent of many phosphatases and it might be possible that it is split off from the phosphatases during autolysis. Moreover the influence of Ca^{++} was studied, to find out whether a non-specific ion-effect also occurs. Table VII gives a survey of the obtained results.

TABLE VII. *Influence of* Mg^{++} *and* Ca^{++} *on the liberation of* P-*compounds from Scenedesmus.* P (PO_4''') and P_{tot}^{water} were determined during autolysis under sterile conditions at room temperature, and expressed as percentage of P_{tot}^{cell} at t = 0. To some samples 1 g/l $MgSO_4 . 7\,H_2O$ or 1 g/l $Ca(NO_3)_2 . 4\,H_2O$ had been added. For experimental data see exp. 9 and 10.

	Experiment 9				Experiment 10			
Added			MgSO$_4$				Ca(NO$_3$)$_2$	
Autolysis during	P(PO_4''')	P_{tot}^{water}	P(PO_4''')	P_{tot}^{water}	P(PO_4''')	P_{tot}^{water}	P(PO_4''')	P_{tot}^{water}
0 h	0%	0%	0%	0%	0%	0%	0%	0%
5 h	33	42	51	58	33	39	30	48
4 d	59	60	71	74	59	60	63	71
15 d	—	72	—	78	—	67	—	72

This table shows that Mg^{++} accelerates the liberation of the P-compounds that are hydrolysed to PO_4''', while Ca^{++} accelerates the liberation of the P-compounds that appear in the medium as P_{bound}^{water}. This might be explained by the supposition that Ca^{++} increases the permeability of the cells. With Mg^{++} no similar effect is observed, possibly through the accelerated hydrolysis to PO_4''', which can leave the cells unhampered.

In the presence of both Mg^{++} and Ca^{++} the extinction of the TCA-w extract (per mg P_{tot}^{susp}) during autolysis decreased (Exp. 9 and 10). This can be explained by assuming, that in the presence of these ions the nucleic acids could not be recovered quantitatively, either through a lowering of the extinction of the nucleic acids (BEAVEN 1955), or through the formation of unsoluble precipitates in the cell.

An indication, that such a precipitate may occur, is the fact that Mg^{++} must be added when nucleophosphate has to be precipitated from a solution, and that Ca^{++} must be added for the isolation of nuclei, by means of which a higher yield of nucleic acids is obtained (DOUNCE 1955). Some investigators assume here an inhibition of autolysis. We are rather inclined to suppose, that during those isolations a – perhaps temporarily – less soluble product is formed.

These experiments are to be repeated, but not before the determination of the nucleophosphates yields completely satisfactory results.

Like the temperature, the Ca^{++} and Mg^{++}-contents of a lake are important ecological factors regarding an eventual autolysis occurring in it.

Influence of NaF

It appeared that F′ has an inhibiting effect on the liberation of P-compounds during autolysis of *Scenedesmus*, which inhibition has its maximum at a concentration of 10^{-2} M. NaF (exp. 11, 12 and 13), and is still observed at 60° C (exp. 5).

In higher concentrations this action of NaF decreases, probably because the solubility of the nucleic acids is increased. In accordance with this the P-content of fraction TCA-w decreases (exp. 12 and 13). High concentrations of NaCl had a similar effect only after a longer duration of the experiment (exp. 13).

Especially the liberation of the P-compounds from fraction alc 60 % and fraction TCA-w appears to be inhibited (exp. 11 and 12), which proves, that both these fractions are broken down by means of enzymes, as had already been stated on page 22 for fraction alc 60 %.

From exp. 4 appears that the extent of inhibition (in this experiment 15 % of P^{cell}_{tot}) does not depend on the method of killing the cells, from which follows once more that the processes studied here are not specific for any particular method of killing.

The influence of NaF on the course of the extinction of the TCA-w extract during autolysis (exp. 11 and 12) can not be explained, before the action of Mg^{++} and Ca^{++} has been elucidated, because it is quite possible that NaF counteracts the influence of these ions by blocking them.

Liberation and mineralisation of N from Scenedesmus quadricauda

a. *Under sterile conditions*

In the previous paragraph was shown, that 70–80 % of the P-compounds leave *Scenedesmus* cells during autolysis under sterile conditions in a few days. Of the N-compounds on the other hand only 20–30 % are liberated.

We applied the same method of fractionation for N- and for P-compounds, which is unusual for N-compounds, but justified in our case by the results. For, it appeared that during autolysis the N-compounds of fraction alc 60 % and fraction TCA-c (i.e. the N-compounds present in the cell in dissolved state) leave the cells. The N-compounds of fraction TCA-w only do so in part; the remaining part belongs without doubt to the nucleic acids (5–10 % of N^{cell}_{tot}). The bulk of the N-compounds, that can not be extracted with ethanol 60 % or with a 5 % TCA solution at 4° C and 90° C, remains also

in the cells (fraction rest-N, 70–80 % of N^{cell}_{tot}). These are the proteins which form about 50 % of the dry weight of the cell.

The nitrogen of nucleic acids and proteins can only be restored to the cycle of elements through the influence of other organisms.

See for experimental data exp. 4, 5, 6, 8, 16 and 17.

b. *Under influence of bacteria*

We have tried to digest the N-slag – remaining after sterile autolysis – by means of bacteria. To this purpose *Scenedesmus* cells were killed with chloroform, left to stand with water during one week and finally washed carefully, all under sterile conditions, to remove all dissolving N-compounds. These cells, which from now on will be referred to as leached cells, were suspended in lake water, containing no detectable amounts of nitrogen or phosphorus, the concentration of N^{cell} being 0.10 mg per ml.

After 5 days 45 % of the added N^{cell} appeared in the solution as NH_3, after 7 days 50 %; then this quantity remained constant. Of the *Scenedesmus* cells only a detritus was left, which could not be identified microscopically. Addition of 0.05 mg of KH_2PO_4/ml to the suspension of leached cells in lake water had a somewhat delaying influence on the production of NH_3. After addition of glucose no production of NH_3 was observed.

From this type of experiments it can not be concluded whether the amount of nitrogen, not converted to NH_3, is still in the *Scenedesmus* cells, or has been consumed by bacteria, because bacteria and *Scenedesmus* residue clot together so strongly that they can not be separated.

Therefore the inhibiting effect of glucose can not be elucidated, as it might be due to an increased bacterial growth, but also to a decreased digestion of *Scenedesmus*. An indication in this direction is the fact, that in the presence of glucose a larger amount of *Scenedesmus* cells remains identifiable.

Moreover the obtained results were rendered unreliable by the growth of ciliates. Therefore in a second experiment the leached cells were suspended in filtered lake water, which had been heated to 45° C during one hour. The growth of ciliates was strongly suppressed by this, while the clotting was rendered less intensive.

25 % of the added N^{cell} was now recovered as NH_3. Besides this about 50 % of the total nitrogen was present in the supernatant after the cells had been centrifuged off. This was due to the fact that in this experiment the *Scenedesmus* cells had been digested largely, and the remaining smaller particles of the *Scenedesmus* detritus were present in the supernatant together with the bacteria, as was confirmed by microscopic examination. Because of this fact the ratio between the nitrogen in *Scenedesmus* detritus and in bacteria could once more not be determined. The sediment contained about 20 % of the nitrogen and practically no bacteria.

As experiments of this type appear to present to many technical

difficulties, we investigated the action of bacteria in pure cultures on the decomposition of the algal cells.

As the slag to be digested consists chiefly of proteins, we chose bacteria which excrete proteolytic enzymes. Our choice fell on *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Proteus vulgaris* and *Bacillus subtilis;* the proteinases of those bacteria have been studied extensively a.o. by Gorini (1950–51) and Haines (1931, 1932, and 1933).

When to this types of bacteria proteins are given as a substrate they may either be hydrolysed to amino acids, which are incorporated immediately by the growing bacteria, or be broken down by oxidation under formation of NH_3. This can be incorporated as long as the bacteria grow, but is liberated when bacterial growth is inhibited, e.g. by external circumstances or special poisons.

We have tried to achieve oxidative breakdown with liberation of NH_3 as follows:

The four previously mentioned bacteria were cultured on bactopeptone, centrifuged, washed with and finally suspended in a solution containing all the salts that are added to peptone water.

Peptone water: NaCl 5 g/l; K_2HPO_4 0.5 g/l; $MgSO_4 . 7H_2O$ 0.1 g/l; $CaCl_2$ or $Ca(NO_3)_2 . 4H_2O$ 0.1 g/l; NaOH to pH = 7.5.

To this suspension leached algae were added, by which the uptake of O_2 and the release of CO_2 of *Proteus vulgaris* and *Bacillus subtilis* increased a little, while those of *Pseudomonas fluorescens* and *Ps. aeruginosa* increased to 5–6 times the original value. Adding of dinitrophenol (DNP, 5×10^{-4} M.) caused a slightly greater increase. After some hours the exchange of gases returned to the original level. A production of NH_3 could, however, never be demonstrated.

In the second place we have tried to obtain bacterial growth on a medium with leached algae as the only source of nitrogen. This has not yet succeeded, not even when a concentrated suspension – cultured on bactopeptone – was added.

More promising results were obtained after partial hydrolysis of the algal proteins with alkali to prepare an "algal peptone".

Scenedesmus cells were extracted with ethanol 60 % and a 5 % TCA solution at 4° C, to remove all soluble substances, and subsequently suspended in twice their own volume of 0.25 N NaOH at 30° C during 24 hours. The cells were centrifuged off and washed with twice their volume of water, which was added to the NaOH extract. This diluted extract was filtered, first through a Schott G 4 and then through a Schott G 5 filter, after which the pH was brought to 7.5 by addition of HCl. The concentration of NaOH had been calculated so as to give the resulting NaCl the concentration, needed for the culture solution of the bacteria (0.5 %). After addition of 0.5 g of K_2HPO_4, 0.1 g of $MgSO_4 . 7 H_2O$ and 0.1 mg of $CaCl_2$ per liter, the solution was sterilised, during which process a precipitate (probably proteins) was formed.

On this medium *Proteus vulgaris* and *Bacillus subtilis* did not grow, while *Pseudomonas aeruginosa* and *Ps. fluorescens* grew very well, causing the precipitate to disappear.

Part of the bacterial suspension cultured on algal peptone was added to a suspension of leached algae. Although the number of the bacteria clearly increased, we did not yet succeed in demonstrating a decrease of the N-content of the *Scenedesmus* cells.

Mineralisation of Si from Stephanodiscus Hantzschii

We will now discuss some preliminary experiments, bearing upon the mineralisation of silicium (exp. 21, 22 and 23).

The mineralisation of Si-compounds proceeds slowly. After killing of the diatoms with chloroform only 0.5–1.5 % of the bound Si is liberated in 1–2 days. This amount increases to 20–30 % only after several weeks, and the process is certainly not finished after 5 weeks. As the rate of mineralisation can, therefore, be dependent only on the hydrolysis rate of the bound Si, or on the dissolution rate of the formed product, we investigated the influence of the pH.

In exp. 22 adding of 0.1 g of $NaHCO_3$ per liter appeared to have no effect, possibly because the, already rather high, pH of the suspension (pH = 7) was not altered. In exp. 23 the mineralisation was clearly increased by the addition of 0.5 g/l of $NaHCO_3$ by which the pH of this suspension had increased from 6.4 to 7.8.

After 3 weeks samples of both suspensions of exp. 23 were filtered off and resuspended in water and in a $NaHCO_3$ solution (0.5 g/l). 2 weeks later the quantity of silicium mineralised in the old and new suspensions was determined. It appeared then, that in the second suspension in water as much silicium had been dissolved as in the first one, but that in the second suspension in a $NaHCO_3$ solution considerably more silicium had been dissolved, than in the first one. We feel justified to conclude, that the liberation of silicium in water is not limited by the solubility, whereas this might be the case in the suspensions with $NaHCO_3$. It will therefore be necessary to renew the suspending fluid regularly when these experiments will be further continued.

In exp. 23 the amount of silicium, still present in the cells after 3 weeks, was also determined, to find out whether Si-compounds, which do not react with the molybdate reagent, are dissolved. This is not the case, for the sum of the silicium, dissolved and present in the cells, is equal to the originally added quantity.

Mineralisation of Fe from scenedesmus quadricauda

A few experiments were carried out to investigate the liberation of iron during autolysis of *Scenedesmus* under sterile conditions. In preliminary experiments no iron could bedem onstrated in the suspension medium. As this could be due to precipitation of alkaline ironphosphate, caused by the relatively high pH of the solutions (pH = 5.5) and the large amount of phosphate present (mg P^{susp}_{tot}/ mg Fe^{susp}_{tot} = 20–30), we added EDTA to the autolysing suspensions (1 mg of Na_2EDTA per 10 ml) to convert the liberated iron to a stable complex. Moreover, as the PO_4''' disturbs the determination of iron and perhaps even the formation of the complex with EDTA, it was

removed for the greater part by transferring the cells to an EDTA solution after a 24 hours' autolysis in water. It appeared than that in one experiment 65 % of the iron present had been dissolved after 47 days, in another one 50 % after 10 days (Fe^{susp}_{tot} was resp. 2 and 3mg/l).

Liberation and mineralisation from other organisms

Finally we investigated whether the results, so far obtained, are restricted to *Scenedesmus* cells or are of a more general importance. Therefore the following organisms were killed with chloroform to observe the liberation and mineralisation of N- and P-compounds: *Ankistrodesmus falcatus (Corda) Ralfs* (exp. 18), *Stephanodiscus Hantzschii Grun.* (exp. 21, 22 and 23), and *Cladophora glomerata (L.) Kuetzing* (exp. 19 and 20).

We need not describe these results in detail. An exception has to be made for exp. 19 and 20. In exp. 19 the large amount of P^{water}_{bound} liberated during autolysis is striking. This sample was collected from a ditch, as the organism is not cultured in the laboratory. In exp. 20 a sample was used that had been collected on the same spot one week later. In the meantime a large amount of Vecht-water, rich in phosphate, had entered the ditch, with the result, that the composition of this sample was different, as appeared from the amounts of PO_4''' in the TCA-w extracts, which had been kept at 90° C during 1 hour. The large amount of P-compounds that are easily hydrolysed in the second sample indicates a high polyphosphate content.

From laboratory experiments it appears also, that addition of phosphate to a P-deficient culture leads to an important increase of the non-nucleophosphate of fraction TCA-w.

In these circumstances the percentages of $P(PO_4''')$ and P^{water}_{bound} liberated during autolysis do not differ from those obtained with *Scenedesmus*.

The addition of $NaHCO_3$ (0.5 g/l) in exp. 23 had an accelerating influence on the liberation of the P-compounds, but did not cause an increase of the total amount. As it has been observed that a change in the pH does not alter the liberation rates of the P-compounds it must be assumed that this accelerating effect is caused by the increase in concentration of the anions present.

Experimental

EXP. 1. *Influence of the method of killing on the liberation of* P-*compounds from Scenedesmus quadricauda.*

Scenedesmus was cultured at 20° C, as has been described in Chapter II. A culture of 4–8 weeks, in which growth had stopped, was centrifuged off, washed several times with H_2O and finally suspended in H_2O. — Two samples (200 ml), A and B, were irradiated in a sterile room in an open glass dish (ø 25 cm) during 4 hours, with the U.V. light of three Philips T.U.V. lamps (30 Watt), mounted 10 cm

above the suspension. Sample C was diluted with sterile H_2O, after which 5 ml of chloroform per liter suspension was added, and was shaken during 15 minutes. Sample C was diluted with sterile H_2O and killed with formaldehyde (1 %). All samples were brought up to the same final volume as accurately as possible. In the four suspensions P^{cell}_{tot} at t = 0 (equal to P^{susp}_{tot}) was determined. — All actions were carried out under sterile conditions at room temperature. — At different moments $P(PO_4''')$ and P^{water}_{tot} were determined in the filtrates of the suspensions. Results as percentage of P^{susp}_{tot}.

Cells killed by	irradiation		irradiation		chloroform		formaldehyde	
P^{susp}_{tot} (mg/l)	4.5		4.9		4.7		4.9	
Autolysis during (*days*)	$P(PO_4''')$	P^{water}_{tot}	$P(PO_4''')$	P^{water}_{tot}	$P(PO_4''')$	P^{water}_{tot}	$P(PO_4''')$	P^{water}_{tot}
0	0%	0%	0%	0%	0%	0%	0%	0%
1	42	64	41	62	53	66	20	28
2	52	69	56	64	63	71	45	52

EXP. 2. *Influence of the density of the suspension and the method of killing on the liberation of P-compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared and divided as in exp. 1. The cells were killed: in sample A by saturation with chloroform, in sample B in the same way after dilution 1:5, in sample C by saturation with toluene. — The remaining sample was used for the fractionation of the P-compounds, as has been described in chapter III. — In all suspensions P^{susp}_{tot}(= P^{cell}_{tot} at t = 0) was determined. — Autolysis at room temperature. — All actions under sterile conditions. — Results as percentage of P^{susp}_{tot}.

Sample	Cells killed by	Autolysis during (*days*)	$P(PO_4''')$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c} + P^{cell}_{TCA\text{-}w}$	Recovered	P^{susp}_{tot} mg/l
D	—	—	0%	0%	29%	70% [1]	99%	—
A	chloroform	1	52	55	3	43	101	4.9
		6	68	74	—	—	—	
B	chloroform	1	50	54	4	41	99	1.1
C	toluene	1	32	35	6	53	94	4.8
		6	69	72	—	—	—	

[1]) at t = 0: $P^{cell}_{TCA\text{-}c}$ = 8 %.

EXP. 3. *Influence of the method of killing and of* NaF *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared, as in exp. 1, and divided into 6 samples. In each sample P^{susp}_{tot} was determined, after which the cells were killed: in sample A by U.V. irradiation from t = 0 till t = 4, as in exp. 1; in sample B by addition of 5 ml of chloroform per liter at t = 2; in sample C by addition of 1 ml of carbon tetrachloride per liter at t = 2; in sample D as in sample A, 10^{-2} M. NaF being present; in sample E as in sample B, 10^{-2} M. NaF being present; the remaining sample F was used for the fractionation of the P-compounds. — Autolysis at room temperature. — Results as percentage of P^{susp}_{tot}. — All actions under sterile conditions.

Sample	Cells killed by	Autolysis during (*days*)	$P(PO_4''')$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$	Recov-ered	P^{susp}_{tot} mg/l
F	—	—	0%	0%	54%	15%	30%	99%	
A	irradiation	1	38	45	—	—	—	—	8.7
		3	—	51	6	3	39	99	
B	chloroform	1	53	55	—	—	—	—	7.5
		3	—	59	10	3	34	106	
C	carbon tetrachloride	1	32	38	—	—	—	—	7.5
		3	—	54	4	3	36	97	
D	irradiation + NaF	1	9	19	—	—	—	—	8.0
		3	—	24	6	4	71	105	
E	chloroform + NaF	1	9	19	—	—	—	—	7.8
		3	—	22	8	5	66	101	

EXP. 4. *Influence of the temperature and of* NaF *on the liberation of* P- *and* N-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into five samples: A_1 was killed by a 4 hours' U.V. irradiation as in exp. 1; A_2 as A_1, 10^{-2} M NaF being present per liter; B_1 with chloroform; B_2 as B_1, 10^{-2} M NaF being present per liter. — The four samples were divided into three equal parts, which were rapidly brought resp. to 0° C, 20° C and 30° C. Sample C was used for the fractionation of the P- and N-compounds. — All actions under sterile conditions.

$P_{tot}^{susp} = 6.9$ mg/l; $N_{tot}^{susp} = 44$ mg/l; mg N/ mg P = N/P = 6.4.

A) P-*compounds.*

Autolysis at	Liberated $P(PO_4''')$ in % of P_{tot}^{susp}																	
	0° C						20° C						30° C					
during (*days*)	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2
0	0%	0%	*0%*	0%	0%	*0%*	0%	0%	*0%*	0%	0%	*0%*	0%	0%	*0%*	0%	0%	*0%*
1	21	10	*11*	32	13	*19*	32	16	*16*	48	27	*21*	36	18	*18*	63	43	*20*
4	31	16	*15*	41	22	*19*	43	24	*19*	59	44	*15*	—	33	-	75	62	*13*

Autolysis at	Liberated P_{tot}^{water} in % of P_{tot}^{susp}																	
	0° C						20° C						30° C					
during (*days*)	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2	A_1	A_2	A_1-A_2	B_1	B_2	B_1-B_2
0	0%	0%	*0%*	0%	0%	*0%*	0%	0%	*0%*	0%	0%	*0%*	0%	0%	*0%*	0%	0%	*0%*
1	45	32	*13*	48	29	*19*	61	48	*13*	65	45	*20*	71	56	*15*	75	67	*8*
4	57	43	*14*	59	37	*12*	74	61	*13*	74	63	*11*	—	70	—	85	77	*8*

Autolysis at	$P_{TCA\text{-}c}^{cell}$ [1]) + $P_{TCA\text{-}w}^{cell}$ in % of P_{tot}^{susp}								P_{rest}^{cell} in % of P_{tot}^{susp}							
	0° C				20° C				0° C				20° C			
during (*days*)	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2
0	66%	66%	66%	66%	66%	66%	66%	66%	8%	8%	8%	8%	8%	8%	8%	8%
4	40	44	33	53	21	29	21	29	6	12	12	—	11	11	10	12

EXP 4 *(Continued)*

Autolysis at during *(days)*	Recovered in % of P^{susp}_{tot}							
	0° C				20° C			
	A_1	A_2	B_1	B_2	A_1	A_2	B_1	B_2
0	97%	97%	97%	97%	97%	97%	97%	97%
4	103	99	104	—	106	101	105	104

[1]) At $t = 0$ $P^{cell}_{TCA\text{-}c}$ amounted to 24 %; after autolysis the amount was so small, that it has been determined together with $P^{cell}_{TCA\text{-}w}$. At $t = 0$ P^{cell}_{alc} amounted to 23 %; after autolysis to less than 1 %.

B) N-*compounds*

The quantity of N-compounds, liberated in the suspensions to which NaF had been added (A_2 and B_2) was equal to that, liberated in the suspensions without NaF (A_1 and B_1). Only the results of autolysis in suspensions A_1 and B_1 are given.

Autolysis at during *(days)*	N^{water}_{tot} in % of N^{susp}_{tot}				$N^{cell}_{TCA\text{-}w}$ in % of N^{susp}_{tot}				N^{cell}_{rest} in % of N^{susp}_{tot}				Recovered in % of N^{susp}_{tot}			
	0° C		20° C		0° C		20° C		0° C		20° C		0° C		20° C	
	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1	A_1	B_1
0	0%	0%	0%	0%	10%	10%	10%	10%	79%	79%	79%	79%	101%	101%	101%	101%
4	16	15	21	20	7	7	7	6	80	86	82	81	96	101	103	101

$N^{cell}_{TCA\text{-}c}$ was before autolysis 2 %, after autolysis less than 1 %.
N^{celll}_{alc} was before autolysis 10 %, after autolysis less than 1 %.

EXP. 5. *Liberation of* N-*compounds and influence of temperature and* NaF *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into 6 samples. The cells were killed: in sample A, B and C with chloroform, at resp. 20° C, 30° C and 60° C; in sample D by heating to 60° C; in sample E with chloroform at 60° C after adding 10^{-2} M. NaF. — During further autolysis these temperatures were maintained. — The remaining sample was used for the determination of P^{susp}_{tot} and N^{susp}_{tot} and for the fractionation of N- and P-compounds. — All actions under sterile conditions. — The results for the P-compounds are given as percentage of P^{susp}_{tot} (a); for the N-compounds (only sample A) as percentage of N^{susp}_{tot} (b). P^{susp}_{tot} = 4.2 mg/l; N^{susp}_{tot} = 27 mg/l; N/P = 6.5.

A) *Librration of* P-*compounds.*

	$P(PO_4''')$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c} + P^{cell}_{TCA\text{-}w}$		P^{cell}_{rest}	Recov-ered
Beginning of experiment:							
	0%	0%	28%	72%	(9%) + (63%)	4%	104%
Autolysis with chloroform at 20° C during:							
5 h	43	49	—	—		—	—
2 d	50	50	8	27	(5) + (22)	—	—
6 d	62	65	8	19	— —	11	103
28 d	65	70	—	—		—	—
Autolysis with chloroform at 30° C during:							
5 h	50	58	—	—		—	—
2 d	62	68	6	18	(4) + (14)	—	—
6 d	73	75	6	10	— —	10	101
Autolysis with chloroform at 60° C during:							
5 h	51	57	—	—		—	—
2 d	69	74	6	13	(1) + (12)	—	—
6 d	73	76	4	10	— —	6	96
Autolysis at 60° C during:							
5 h	48	—	—	—		—	—
2 d	63	68	6	14	(1) + (13)	—	—
6 d	66	71	4	10	— —	12	97
Autolysis with chloroform and 10^{-2} M. NaF *at 60° C during:*							
5 h	18	—	—	—		—	—
2 d	37	60	10	20	(2) + (18)	—	—
6 d	45	72	5	16	— —	13	106

B) *Liberation of* N-*compounds.*

	N^{water}_{tot}	N^{cell}_{all}	$N^{cell}_{TCA\text{-}c} + N^{cell}_{TCA\text{-}w}$		N^{cell}_{rest}	Recov-ered
Beginning of experiment:						
	0%	6%	7%	(0%) + (7%)	86%	99%
Autolysis with chloroform at 20° C during:						
6 d	10	4	10	— —	80	104
28 d	25	—	—		70	—

EXP. 6. *Liberation of* N- and P-*compounds from Scenedesmus quadricauda.*

A 12 days' old culture of *Scenedesmus* was treated as sample A, exp. 5. — $P_{tot}^{susp} = 5.4$ mg/l; $N_{tot}^{susp} = 36$ mg/l; N/P = 6.7.

All actions under sterile conditions.

A) *Liberation of* P-*compounds.*

Autolysis during	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}+P_{TCA\text{-}w}^{cell}$		P_{rest}^{cell}	Recovered
0 h	0%	0%	27%	67%	(11%)+(56%)	3%	97%
5 h	51	65	—	—		—	—
2 d	63	74	1	20	(3) (17)	—	—
6 d	74	80	1	10	— —	8	99
28 d	76	81	—	—		—	—

B) *Liberation of* N-*compounds*

Autolysis during (*days*)	N_{tot}^{water}	N_{alc}^{cell}	$N_{TCA\text{-}c}^{cell}+N_{TCA\text{-}w}^{cell}$		N_{rest}^{cell}	Recovered
0	0%	12%	4%	(0%) + (4%)	82%	98%
6	14	7	6	— —	72	99
28	20	—	—		72	—

EXP. 7. *Liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1, and killed with chloroform at room temperature. — Further data as exp. 6. — All actions under sterile conditions. — $P_{tot}^{susp} = 6.2$ mg/l.

Autolysis during	$P(PO_4''')$	P_{bound}^{water}	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recovered
0 h	0%	0%	0%	25%	32%	38%	4%	99%
3.5 h	18	10	28	—	—	—	—	—
6 h	29	14	43	—	—	—	—	—
1 d	48	12	60	3	2	36	—	—
5 d	65	3	68	2	2	25	—	—
12 d	68	4	72	1	2	26	3	104

EXP. 8. *Liberation of* N-*compounds and influence of the temperature on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into four samples. The cells were killed: in sample A, B and C with chloroform at resp. 4° C, 20° C and 30° C. During further autolysis these temperatures were maintained. — The remaining sample was used for the determination of N_{tot}^{susp} and P_{tot}^{susp}, and for the fractionation of the N- and P-compounds. — Of the TCA-w extracts the extinction was determined at 260 mμ, and $E_{TCA\text{-}w}^{cell}$ (the extinction per mg P_{tot}^{susp} per ml per 1 cm cuvette) calculated. — Results for the

P-compounds as percentage of P_{tot}^{susp} (a), for the N-compounds as percentage of N_{tot}^{susp} (b). — All actions under sterile conditions.
$P_{tot}^{susp} = 8.8$ mg/l; $N_{tot}^{susp} = 72$ mg/l; N/P = 8.2.

A) *Liberation of* P-*compounds.*

	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recovered	$E_{TCA\text{-}w}^{cell}$
Beginning of experiment:								
	0%	0%	22%	10%	60%	5%	97%	71
Autolysis with chloroform at 4° C during:								
1 d	21	24	9	6	50	—	—	71
5 d	—	26	9	6	50	—	—	72
14 d	—	30	8	7	51	4	100	71
Autolysis with chloroform at 20° C during:								
1 d	32	34	7	7	52	—	—	70
5 d	—	43	7	7	32	—	—	71
14 d	—	55	8	4	25	3	95	72
Autolysis with chloroform at 30° C during:								
1 d	33	38	9	8	40	—	—	65
5 d	—	62	6	2	20	—	—	71
14 d	—	63	5	0	26	5	99	74

B) N-*compounds.*

	$N(NH_4^+)$	N_{tot}^{water}	N_{alc}^{cell}	$N_{TCA\text{-}c}^{cell} + N_{TCA\text{-}w}^{cell}$		
Autolysis with chloroform at 20° C during:						
0 d	0%	0%	10%	17%	(10%) +	(7%)
1 d	5	11	4	13	—	—
5 d	6	11	1	14	—	—
14 d	4	10	2	14	—	(9)

EXP. 9. *Influence of* Mg^{++} *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into three samples. The cells were killed: in sample A with chloroform, in sample B with chloroform, after 1 g of $MgSO_4 . 7\ H_2O$ per liter had been added. — The remaining sample was used for the determination of P_{tot}^{susp} and for the fractionation of the P-compounds. — Autolysis at room temperature. — All actions under sterile conditions. — Results as percentage of P_{tot}^{susp}. For the determination of $E_{TCA\text{-}w}^{cell}$ vide exp. 8. — $P_{tot}^{susp} = 4.0$ mg/l; N/P = 6.5.

	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$		P_{rest}^{cell}	Recov-ered	$E_{TCA\text{-}w}^{cell}$
					$P(PO_4''')$	P_{bound}			
Beginning of experiment:									
	0%	0%	25%	12%	26%	28%	8%	99%	52
Autolysis with chloroform during:									
5 h	33	42	—	—	—	—	—	—	—
4 d	59	60	6	6	4.4	15.4	8	100	51
15 d	—	72	—	—	—	—	—	—	—
Autolysis with chloroform in the presence of $MgSO_4$ *during:*									
5 h	51	58	—	—	—	—	—	—	—
4 d	71	74	5	3	2	10	7	101	37
15 d	—	78	—	—	—	—	—	—	—

EXP. 10. *Influence of* Ca^{++} *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was treated as in exp. 9, but sample B was killed with chloroform after addition of 1 g of $Ca(NO_3)_2 \cdot 4\,H_2O$ per liter. — For further data vide exp. 9. — All actions under sterile conditions. — $P_{tot}^{susp} = 3.8$ mg/l; N/P = 8.

	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$		P_{rest}^{cell}	Recov-ered	$E_{TCA\text{-}w}^{cell}$
					$P(PO_4''')$	P_{bound}			
Beginning of experiment:									
	0%	0%	33%	13%	24%	33%	0%	103%	52
Autolysis with chloroform during:									
5 h	33	39	—	—	—	—	—	—	—
4 d	59	60	5	4	4	17	7	97	52
15 d	—	67	—	—	—	—	—	—	—
Autolysis with chloroform in the presence of $Ca(NO_3)_2$ *during:*									
5 h	30	48	—	—	—	—	—	—	—
4 d	63	71	2	7	5	14	5	104	38
15 d	—	72	—	—	—	—	—	—	—

EXP. 11. *Influence of* NaF *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension, prepared as in exp. 1, was divided into six samples. The cells were killed with chloroform: in sample A, B, C, D and E after addition of resp. 0, 1, 4, 40 and 400 mg/l of NaF. The remaining sample was used for the determination of P_{tot}^{susp} and for the fractionation of the P-compounds. — Autolysis at room temperature. — All actions under sterile conditions. — For the determination of $E_{TCA\text{-}w}^{cell}$ vide exp. 8. — Results as percentage of P_{tot}^{susp}.

$P_{tot}^{susp} = 5.4$ mg/l; N/P = 9.4.

A) *Influence of* NaF *on liberation of* P (PO_4''') *and of* P_{tot}^{water}.

NaF mg/l Autolysis during	0		1		4		40		400	
	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
5 h	33	51	30	46	27	46	30	40	14	25
3 d	47	58	—	—	—	—	40	52	23	32

B) *Relation between the concentrations of* NaF *added and of the* P-*compounds of the different fractions.*

	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recov-ered	$E_{TCA\text{-}w}^{cell}$
Beginning of experiment:								
	0%	0%	29%	27%	38%	8%	102%	74
Autolysis during 3 days with NaF:								
0 mg/l	47	58	3	9	19	7	96	44
40 mg/l	40	52	5	9	23	5	94	48
400 mg/l	23	32	15	17	27	6	97	67

EXP. 12. *Influence of* NaF *on the liberation of* P-*compounds from Scenedesmus quadricauda.*
A *Scenedesmus* suspension was prepared as in exp. 1, and divided into five samples. The cells in samples A, B, C and D were killed with chloroform, after addition of resp. 0, 400, 1200 and 4000 mg of NaF per liter. — The remaining sample was used for the determination of P_{tot}^{susp} and for the fractionation of the P-compounds. Autolysis at room temperature. All actions under sterile conditions. For further experimental data vide exp. 11. — P_{tot}^{susp} = 11 mg/l; N/P = 9.0.

	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recov-ered	$E_{TCA\text{-}w}^{cell}$
Beginning of experiment:								
	0%	0%	32%	19%	42%	4%	97%	100
After a 3 days' autolysis with chloroform:								
0 mg NaF/l	58	59	5	7	21	10	102	59
400 mg NaF/l	26	32	15	7	33	11	98	92
1200 mg NaF/l	21	31	15	9	32	10	97	83
4000 mg NaF/l	21	46	16	11	15	10	98	40
After a 7 days' autolysis with chloroform:								
0 mg NaF/l	67	67	3	4	21	7	102	—
1200 mg NaF/l	30	49	10	5	35	5	104	—

EXP. 13. *Influence of* NaF *and* NaCl *on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into 8 samples. The cells were killed: in sample A–F with chloroform, after addition of resp. 0, 0.13, 0.48, 1, 4 and 10 g of NaF per liter; in sample G with chloroform after addition of 15 g of NaCl per liter. The remaining sample was used for the determination of P_{tot}^{susp}. — Autolysis at room temperature. — All actions under sterile conditions. — $P_{tot}^{susp} = 6.5$ mg/l; N/P = 6.3.

Autolysis during (*days*)	NaF (mg/l) added												NaCl added	
	0		0.13		0.48		1		4		10		15 mg/l	
	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
2	47	59	12	24	10	21	11	24	18	43	17	66	50	60
4	58	69	22	37	21	39	22	44	29	61	21	70	61	78

EXP. 14. *Effect of a preceding cold treatment on the liberation at 20° C of the* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared as in exp. 1 and divided into two samples. The cells were killed with chloroform: in sample A at 0° C; autolysis proceeded at the same temperature—parts of the suspension, A_2, A_3 and A_4, were placed at 20° C after resp. 1, 3 and 10 days-; in sample B at 20° C; autolysis proceeded at the same temperature. — $P(PO_4''')$ and P_{tot}^{water} were determined in the filtrates and given as percentage of P_{tot}^{susp}. — All actions under sterile conditions. — $P_{tot}^{susp} = 5.0$ mg/l; N/P = 13.6.

Sample		A		A_2		A_3		A_4		B	
Autolysis during (*days*)		$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
		autolysis at 0° C		*autolysis at 0° C*		*autolysis at 0° C*		*autolysis at 0° C*		*autolysis at 20° C*	
	0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	1	29	39	29	39	29	39	29	39	50	55
1								*autolysis at 20° C*			
	2	36	43	36	43	36	43	54	61	58	62
	3	—	—	—	—	—	—	59	64	59	63
3						*autolysis at 20° C*					
	5	—	—	—	—	60	63	—	—	66	66
10				*autolysis at 20° C*							
	12	45	49	65	65	61	68	—	—	—	—

EXP. 15. *Influence of the temperature on the liberation of* P-*compounds from Scenedesmus quadricauda.*

A *Scenedesmus* suspension was prepared and killed by irradiation at 20° C, as described in exp. 1. Afterwards the suspension was divided into four samples, which were placed resp. at 2° C, 10° C, 20° C and 30° C. — $P(PO_4''')$ and P_{tot}^{water} were determined in the filtrates and given as percentage of P_{tot}^{susp}. — Results as percentage of P_{tot}^{susp}. — All actions under sterile conditions.
$P_{tot}^{susp} = 3.0$ mg/l; N/P = 9.8.

Autolysis at during (*days*)	2° C		10° C		20° C		30° C	
	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}	$P(PO_4''')$	P_{tot}^{water}
0	0%	0%	0%	0%	0%	0%	0%	0%
1	12	16	13	17	22	33	41	56
2	20	26	18	27	32	51	46	66
3	23	30	23	30	40	58	51	68
4	23	32	23	35	41	61	50	65
6	28	38	32	45	41	58	54	67
14	37	54	35	49	49	56	59	75

EXP. 16. *Liberation of* N- *and* P-*compounds from Scenedesmus quadricauda.*

To a *Scenedesmus* culture phosphate was added. After 24 hours a suspension was prepared as in exp. 1. — The cells were killed with chloroform at room temperature, after N_{tot}^{susp} and P_{tot}^{susp} had been determined, and the fractionation of the N- and P-compounds had been carried out in an aliquot. — The autolysing suspension was kept at room temperature for 63 days. Results for the P-compounds as percentage of P_{tot}^{susp} (a), for the N-compounds as percentage of N_{tot}^{susp}. (b). — All actions under sterile conditions.
$P_{tot}^{susp} = 53.5$ mg/l; $N_{tot}^{susp} = 177$ mg/l; N/P = 3.3.

A) *Liberation of* P-*compounds.*

Autolysis during (*days*)	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recovered
0	0%	9%	5%	82%	2%	98%
4	76	0	3	18	1	98
18	86	0	0	12	1	99
36	85	0	0	16	1	102
63	89	1	1	7	1	99

B) *Liberation of* N-*compounds.*

Autolysis during (*days*)	N_{tot}^{water}	N_{alc}^{cell}	$N_{TCA\text{-}c}^{cell}$	$N_{TCA\text{-}w}^{cell}$	N_{rest}^{cell}	Recovered
0	0%	5%	2%	8%	82%	97%
4	8	1	2	6	81	98
18	11	2	1	7	73	94
36	14	2	0	6	70	92
63	17	6	0	7	71	101

EXP. 17. *Liberation of* P-*compounds from Scenedesmus quadricauda.*

In a 10 liter flat bottomed flask 8 liters of *Scenedesmus* suspension were cultured. After a 4, 11 and 31 days' growth two samples were siphoned out; one was used for the fractionation of the P-compounds (A), the other one was washed and killed with chloroform; after a 4 days' autolysis at room temperature P_{tot}^{susp} and the distribution of the phosphate over the different fractions were determined (B). The results appeared to be independent of the age of the culture, therefore only those after an 11 days' growth are given (as percentage of P_{tot}^{susp}). — All actions under sterile conditions.

A) *Fractionation of the* P-*compounds in Scenedesmus quadricauda.*

Growth during (*days*)	P_{alc}^{cell} soluble in $CHCl_3$	P_{alc}^{cell} insoluble in $CHCl_3$	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$ $P(PO_4''')$	$P_{TCA\text{-}w}^{cell}$ P_{bound}	P_{rest}^{cell}	Recov-ered	P_{tot}^{susp} mg/l	N/P
4	12%	11%	7%	32%	32%	3%	97%	1.2	8.4
11	19	20	13	18	30	0	100	1.4	11
31	16	16	20	10	24	7	93	1.8	14

B) *Liberation from Scenedesmus quadricauda after an 11 days' growth.*

Autolysis during (*days*)	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$ $P(PO_4''')$	$P_{TCA\text{-}w}^{cell}$ P_{bound}	P_{rest}^{cell}	Recov-ered
0	0%	0%	39%	13%	18%	30%	0%	100%
4	58	63	1	4	7	16	4	95

EXP. 18. *Liberation of* P- *and* N-*compounds from Ankistrodesmus falcatus.*

Ankistrodesmus was cultured in a 10 l flat bottomed flask. After a 5 weeks' growth a suspension was prepared as in exp. 1. — The cells were killed with chloroform, after N_{tot}^{susp} and P_{tot}^{susp} had been determined and the fractionation of P- and N-compounds had been carried out (Schott glass filter G 5 M) in an aliquot. — Autolysis at room temperature. — All actions under sterile conditions. — Results as percentage of P_{tot}^{susp} and N_{tot}^{susp}.

$P_{tot}^{susp} = 23$ mg/l; $N_{tot}^{susp} = 84$ mg/l;N/P = 3.6.

A) *Liberation of* P-*compounds.*

Autolysis during (*days*)	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$	P^{cell}_{rest}	Recovered
0	0%	16%	2%	79%	3%	100%
4	47	5	1	42	1	96
44	80	0	0	20	2	102

B) *Liberation of* N-*compounds.*

Autolysis during (*days*)	N^{water}_{tot}	N^{cell}_{alc}	$N^{cell}_{TCA\text{-}c}$	$N^{cell}_{TCA\text{-}w}$	N^{cell}_{rest}	Recovered
0	0%	6%	3%	10%	80%	99%
4	9	3	2	6	77	97
44	15	2	0	6	82	105

EXP. 19. *Liberation of* P-*compounds from Cladophora glomerata.*

From a ditch *Cladophora* was collected. This alga was washed with sterile water; all other organisms were removed as well as possible. Only some diatoms and a little amount of Euglena remained, but they certainly did not surpass 1 % of the material. Bacteria were practically not present any more. — Immediately afterwards a sample of the material was drawn and used for the fractionation of the P-compounds (Schott glass filter G 5 M), the rest was suspended in 1 l of sterile water and killed with chloroform. — Autolysis at room temperature. — After 7 days the suspension was fractionated again. — Both obtained TCA-w extracts were kept at 90° C for one hour after the cells had been removed, to determine that part of the P-compounds, which is easily hydrolysed. — All actions under aseptic conditions. — Results as percentage of P^{susp}_{tot}, which was calculated by addition of the different fractions. — $P^{susp}_{tot} = 7.3$ mg/l; N/P = 8.4.

Autolysis during	$P(PO_4''')$	P^{water}_{tot}	P^{cell}_{alc}	$P^{cell}_{TCA\text{-}c}$	$P^{cell}_{TCA\text{-}w}$		P^{cell}_{rest}
					$P(PO_4''')$	P_{bound}	
0 h	0%	0%	10%	10%	23%	44%	13%
2 h	—	22	—	—	—	—	—
4 h	—	25	—	—	—	—	—
21 h	12	49	—	—	—	—	—
3 d	26	71	—	—	—	—	—
7 d	48	85	0	1	4	7	3

EXP. 20. *Liberation of* N- *and* P-*compounds from Cladophora glomerata.*

At the same place, but 1 week later than in exp. 19 *Cladophora* was collected again; the ditch had been filled in the meantime with water, rich in PO_4'''. — Further data as in exp. 19. — Moreover the liberation of the N-compounds after a 9 days' autolysis was determined. — $P^{susp}_{tot} = 8.4$ mg/l; $N^{susp}_{tot} = 71$ mg/l; N/P = 8.4

A) *Liberation of* P-*compounds.*

Autolysis during (*days*)	$P(PO_4''')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$		P_{rest}^{cell}
					$P(PO_4''')$	P_{bound}	
0	0%	0%	11%	17%	45%	23%	4%
7	80	88	—	—	—	—	—
9	—	88	0	0	3	10	—

B) *Liberation of* N-*compounds.*

Autolysis during (*days*)	N_{tot}^{water}	N_{alc}^{cell}	$N_{TCA\text{-}c}^{cell}$	$N_{TCA\text{-}w}^{cell}$	N_{rest}^{cell}
0	0%	17%	1%	10%	72%
9	25	2	0	8	65

EXP. 21. *Liberation of* Si- *and* P-*compounds from Stephanodiscus Hantzschii.*

Stephanodiscus was cultured in Fernbach flasks without aeration, as described in chapter II. A suspension of about 5 weeks old cells was prepared as in exp. 1 and divided into two samples. — The cells in sample A were killed with chloroform, after which the liberation of Si- and P-compounds at room temperature was followed up. — Sample B was used for the determination of P_{tot}^{susp}, N_{tot}^{susp} and Si_{tot}^{susp} and for the fractionation of the P-compounds (Schott glass filter G 5 M). — Results as percentage of P_{tot}^{susp} and Si_{tot}^{susp}. — All actions under sterile conditions. P_{tot}^{susp} = 11 mg/l; Si_{tot}^{susp} = 97 mg/l; N_{tot}^{susp} = 33 mg/l; Si/P = 8.9; N/P = 3.1.

Autolysis during (*days*	$Si(SiO_3'')$	P_{tot}^{water}	P_{alc}^{cell}	$P_{TCA\text{-}c}^{cell}$	$P_{TCA\text{-}w}^{cell}$	P_{rest}^{cell}	Recovered
0	0.0%	0%	19%	41%	32%	5%	97%
1	0.5	59	—	—	—	—	—
2	1.6	60	—	—	—	—	—
11	15	66	—	—	—	—	—
21	27	78	1	3	10	4	96

EXP. 22. *Influence of* $NaHCO_3$ *on the liberation of* Si- *and* P-*compounds from Stephanodiscus Hantzschii.*

A suspension of *Stephanodiscus* was prepared as in exp. 21 and divided into three samples. The cells were killed with chloroform in samples A and B after addition of $NaHCO_3$ to sample B. The remaining sample was used for the determination of P_{tot}^{susp}, N_{tot}^{susp} and Si_{tot}^{susp} and for the fractionation of the P-compounds. — Autolysis at room temperature. — All actions under sterile conditions. — P_{tot}^{susp} = 8 mg/l; Si_{tot}^{susp} = 56.5 mg/l; N_{tot}^{susp} = 27 mg/l; Si/P = 7; N/P = 3.3. — P_{alc}^{cell} = 23%; $P_{TCA\text{-}c}^{cell}$ = 28%; $P_{TCA\text{-}w}^{cell}$ = 42%; P_{rest}^{cell} = 7% of P_{tot}^{susp}.

$NaHCO_3$ added	0 mg/l			100 mg/l		
Autolysis during (*days*)	$Si(SiO_3'')$	P^{water}_{tot}	pH	$Si(SiO_3'')$	P^{water}_{tot}	pH
0	0%	0%	7	0%	0%	7
7	2	65	7	2	70	7
14	6	70	7	6	71	7
28	15	—	—	15	—	—
42	19	72	7	18	74	7

EXP. 23. *Influence of* $NaHCO_3$ *on the liberation of* Si-, P- *and* N-*compounds from Stephanodiscus Hantzschii.*

Data as in exp. 22. — After a 3 weeks' autolysis 50 ml of both suspensions (susp. A_1 and A_2) were filtered off and the cells were suspended anew in 50 ml of H_2O and of $NaHCO_3$ solution (susp. B_1 and B_2). — The liberation of Si- and P-compounds was followed up in suspensions A_1, A_2, B_1 and B_2, of N-compounds only in suspensions A_1 and A_2. — Results as percentage of Si^{susp}_{tot}, P^{susp}_{tot} and N^{susp}_{tot}. — All actions under sterile conditions.

$P^{snsp}_{tot} = 20$ mg/l; $N^{susp}_{tot} = 63$ mg/l; $Si^{susp}_{tot} = 132$ mg/l; Si/P = 6.6; N/P = 3.2.
$P^{cell}_{alc} = 19\ \%$; $P^{cell}_{TCA\text{-}c} = 5\ \%$; $P^{cell}_{TCA\text{-}w} = 73\ \%$; $P^{cell}_{rest} = 3\ \%$ of P^{susp}_{tot}.
$N^{cell}_{alc} = 18\ \%$; $N^{cell}_{TCA\text{-}c} = 1\ \%$; $N^{cell}_{TCA\text{-}w} = 12\ \%$; $N^{cell}_{rest} = 65\ \%$ of N^{susp}_{tot}.

$NaHCO_3$ added	0 mg/l						
	suspension A_1				suspension B_1		
Autolysis during (*days*)	$Si(SiO_3'')$	P^{water}_{tot}	N^{water}_{tot}	pH	$Si(SiO_3'')$	P^{water}_{tot}	pH
0	0%	0%	0%	6.4			
7	4	59	—	6.4			
14	8	77	—	—			
21	12	84	20	6.4			
					0%	0.0%	6.4
35	17	76	—	6.4	4	1.5	6.4
70	21	84	28	5.5	7	1.0	5.4

$NaHCO_3$ added	500 mg/l						
	suspension A_2				suspension B_2		
Autolysis during (*days*)	$Si(SiO_3'')$	P^{water}_{tot}	N^{water}_{tot}	pH	$Si(SiO_3'')$	P^{water}_{tot}	pH
0	0%	0%	0%	7.8			
7	11	86	—	7.8			
14	16	86	—	—			
21	20	81	30	7.8			
					0%	0.0%	7.8
35	23	82	—	7.8	17	1.5	7.8
70	25	86	39	7.9	23	1.0	8.2

CHAPTER V

DISCUSSION

In principle the problem of determining the rate of liberation of elements from dying phytoplankton in natural surroundings can be studied with two methods, either by comparing the chemical composition of the dead plankton cells with that of the living ones, or by measuring the quantity of liberated compounds when the algae have died off. The method, first mentioned, was applied by KLEEREKOPER (see page 54). It has the advantage that the results are hardly affected by a possible uptake of the liberated compounds by the living organisms, but it requires either a rather constant composition of the living or the dead plankton, or a thorough investigation into the changes in composition and into the interval between the dying off and collecting of the algae.

The second method yields the best results when all algal cells die off rapidly, which happens rarely in nature; it has the disadvantage that the uptake of the liberated compounds by other organisms is a seriously disturbing factor.

In both methods the mineralisation is determined, as it is effected by the total biocoenosis, which yields a result dependent on many and various factors.

We wished to study the liberation processes during autolysis separately. In order to eliminate disturbing conversions – e.g. a re-uptake either by other organisms or by still living algal cells – we carried out our experiments with pure uni-algal cultures, and killed the cells all at once, which had to be done in such a way that the results were comparable to natural situations. However, very little is known as yet about the circumstances under which algae die off in nature. The numerous causes, that are of no interest within the scope of our investigation, because they do not lead to autolysis, are left out of consideration.

Frequently the dying off of algae is attributed to a depletion of nutrients, but it is questionable whether this is the most important factor. For, in a culture depleted of nutrients many algae can live on for months, provided the light intensity and the temperature are not too high.

In an extensive investigation into the periodicity of *Asterionella* and *Melosira*, LUND found (1949, 1950, 1954 and 1955), that the growth had been stopped by a Si-deficit (1950), and that *Asterionella* died off eventually. He assumed, that the organism divided once more after Si-depletion, but that the cell-walls could no longer be silicified. This supposition does, however, certainly not hold for all diatoms, because all our cultures can live on for months after Si-depletion, provided they are placed in dim light. Lund's observations were not sufficiently supported by laboratory experiments. Especially the joined influence of high light intensity and temperature ought to be investigated.

It is not unlikely that often the dying off of algae is indeed caused

by exposure to the combination of strong light and high temperature, as is mentioned by RODHE (1948).

Consequently we choose irradiation with U.V. light as a way to kill the cells rapidly. It is important that we could demonstrate that almost the same autolytical conversions occurred whether the cells were killed by chloroform treatment, by irradiation with U.V. light or by heating to 60° C. It strengthened our opinion that the results are not restricted to the particular circumstances of our experiments, but might be helpful to elucidate the phenomena observed in nature.

Experiments on the course of autolysis of plankton are reported in literature. STEINER (1938*a*, 1938*b*) killed zoöplankton with chloroform and noted the liberation of phosphate and phosphatases from the cells. WAKSMANN (1937) found, that diatoms, suspended in seawater and placed in the dark, die and give off phosphate to the water. HARVEY (1955) found, that about 40 % of the total amount of P-compounds present in *Skeletonema costatum* was liberated in three hours as P_{bound}^{water} and 35 % as PO_4''', when the cells had been pulverized in the presence of chloroform.

Our experiments resemble most those of HOFFMANN (1951, 1953 and 1956), who killed phytoplankton with chloroform. We find for *Scenedesmus* a liberation rate of the same order of magnitude as reported by him. He fractionated his material before autolysis with hot water, and discerned three fractions: inorganic phosphate, bound phosphate soluble in hot water and insoluble bound phosphate. He calculated that in case of autolysis 70 % of the insoluble bound phosphate leaved the cells, under complete hydrolysis to PO_4'''. He supposed that the soluble bound phosphate present in the cell was liberated without hydrolysis to PO_4''', because he found no decrease of the bound phosphate in the water. We can not share his conclusions, that the insoluble phosphate is hydrolysed to PO_4''' during liberation, while the soluble bound phosphate is not, because they are based on the ratio between the liberated amounts of PO_4''' and bound phosphate. We found in our experiments, that this ratio depends on the methods of killing. We obtained a larger amount of PO_4''' after killing with chloroform than after killing with U.V. light.

70–80 % of the element phophorus leaves the killed *Scenedesmus* cells, in case of autolysis under sterile conditions, in a few days.

The PO_4''', which constitutes 0–5 % of P_{tot}^{cell} and forms a part of the first fraction obtained in the fractionation (fraction alc 60 %), leaves the cells rapidly. Another part of this fraction, the phospholipids (10–20 % of P_{tot}^{cell}), is broken down enzymatically and liberated as PO_4'''.

10–30 % of the P-compounds, originally present in the cells, can be extracted by a 5 % TCA solution at 4° C (fraction TCA-c) from the residue, obtained after removal of the compounds soluble in ethanol 60 %. During autolysis at 0° C this fraction is liberated without hydrolysis to PO_4''', at 20° C they are partly hydrolysed to PO_4'''. It is not unlikely, that the rate at which these compounds

diffuse out of the cells is limited by the membranes of the cell. The stimulating effect of Ca-ions on the liberation of these compounds points in this direction. Moreover, the observation that at 0° C these compounds leave the cells in H_2O in 48 hours, but in a 5 % TCA solution in 1 hour, indicates a limited diffusion. It is remarkable, that the liberation in water at 0° C can happen after the cells have been boiled with ethanol 60 %, which therefore evidently does not abolish the limitation of the diffusion. The enzymes, however, are destroyed, from which follows, that the liberation of these compounds can be brought about without enzymes.

The P-compounds, which can still be extracted – after the removal of fraction alc 60 % and fraction TCA-c – with a 5 % TCA solution at 90° C (fraction TCA-w) and constitute 30–70 % of P_{tot}^{cell}, belong to at least two groups: the nucleophosphates, which are not liberated, and the other P-compounds, "polyphosphates" which are liberated, probably under influence of enzymes.

The polyphosphates have, besides the function of energy-storage (vide e.g. WIAME, 1958), a second, ecological, function: that of phosphate-storage. In our experiments it appeared, that P-deficient cells, when phosphate was replenished, could store, in the form of polyphosphate, in one hour three times as much phosphate as was already present in them.

In case of autolysis the P-compounds of the fraction alc 60 % and fraction TCA-c and the polyphosphates are rapidly liberated, thus forming an ecologically important group of substances. The nucleic acids and the protein-phosphates (fraction rest-P, about 5 % of P_{tot}^{cell}) are not liberated. Their phosphate becomes only available for the autotrophic organisms after a bacterial breakdown of the killed cells.

Quite a different picture is found in the liberation and mineralisation of the element nitrogen.

The N-compounds, present in the cell in dissolved state, are rapidly liberated in case of autolysis, but they represent only a small percentage of the nitrogen present in the algae studied. The bulk, the protein-nitrogen, remains as a slag.

This nitrogen can enter the cycle by means of bacterial digestion. We extended our investigations to this phenomenon to acquire a quantitative impression of it. Therefore, "leached" *Scenedesmus* cells – i.e. cells from which the soluble P- and N-compounds had been removed through autolysis and repeated washing – were suspended in filtered lake water at 30° C. After 5 days the *Scenedesmus* cells had desintegrated and 50 % of the added nitrogen had appeared in the medium as ammonia, which amount further remained constant; nitrite and nitrate had not been formed. Microscopically it could be demonstrated that various types of bacteria and ciliates had developed.

The quantity of ammonia liberated can be used as a measure for the digestion by bacteria, when a constant ratio exists between this quantity and that of the N-compounds digested. The latter amount could not yet be determined, because of the strong clotting of *Scenedesmus* detritus and bacteria.

By keeping the lake water at 45° C during one hour the growth of ciliates was strongly suppressed and the number of species of developing bacteria reduced. After this treatment maximally 25 % of the *Scenedesmus* nitrogen was liberated as ammonia; moreover another 50 % of the nitrogen remained in the supernatant, when the *Scenedesmus* cells were centrifuged off. As the supernatant contained small fragments of *Scenedesmus* cells as well as bacteria, the digestion could once more not be measured.

Although this phenomenon may be important for a bacterial putrefaction in a lake – for, as the sedimentation rate of the nitrogen slag is considerably decreased, the slag can stay longer in the upper water layers – it meant a great technical difficulty in laboratory experiments, where we wished to follow the course of the protein digestion quantitatively.

Better results were expected from experiments, in which *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Proteus vulgaris*, which are known to excrete proteinases, were cultured on a medium with leached *Scenedesmus* cells as the only source of nitrogen.

Against expectation this has not succeeded. Both types of *Pseudomonas*, however, could be cultured on a medium with no other source of nitrogen than the N-compounds, present in an alkaline extract of leached cells ("algal peptone"). A protein-precipitate, formed after the pH of the extract was brought to 7.5, disappeared during the growth of the bacteria.

Starting from the supposition, that the inability to culture bacteria on the leached cells themselves is due to the fact that the bacteria are not adapted to the substrate, we tried to subculture bacteria, grown on algal peptone, on leached cells. The results of these experiments are as yet dubious.

A second observation, which justified further investigations in this direction, is the fact, that the same types of *Pseudomonas* also showed a five- to sixfold increase of O_2 uptake and CO_2 release, when leached cells were given to a suspension of bacteria as a substrate, whereas *Bacillus subtilis* and *Proteus vulgaris* did not do so. This increase was, however, not accompanied by a production of ammonia.

This fact can not be caused by growth of the bacteria, as addition of DNP, which increased the exchange of gases by a few per cent, did not alter it. Moreover, when aspartic acid was given as a substrate under exactly similar circumstances, it appeared that 56 % of its nitrogen was converted to ammonia, when no DNP was added, and 71 % when DNP was added.

This ammonia production was strongly inhibited by addition of glucose, which suggested, that perhaps a liberation of carbohydrates from *Scenedesmus* cells prevented the production of ammonia. Although it is improbable, that many carbohydrates are liberated from the leached cells, we have tried to find out, by means of the R. Q., whether carbohydrates, proteins or a combination of both were respired. However, interpretation of the R.Q. is not possible, as appeared from the following observations:

When aspartic acid, succinic acid, malic acid or acetic acid were given to *Pseudomonas fluorescens* and *Ps. aeruginosa* as a substrate, the products of oxidation were not only CO_2 and H_2O, but probably oxalic acid as well. The ratio between combustion and oxalic acid formation appeared not to be the same for the different substrates mentioned. Thus succinic acid yielded 2 Mol O_2, whereas malic acid yielded 1 Mol O_2 per Mol acid. These observations can be explained by the action of the glyoxylic acid cycle in the bacteria (KORNBERG 1958, SAZ 1956).

Summarizing we can mention four possible causes, why no ammonia is produced:

1) Growth of the bacteria, which moreover is inhibited insufficiently by DNP. In view of the results with respiration of aspartic acid this seems improbable.
2) Simultaneous respiration of carbohydrates. This seems also improbable, because the substrate has been washed thoroughly.
3) Insufficient adaptation.
4) Wrong choice of bacteria.

We hope to effect a necessary adaptation by culturing of the bacteria on algal peptone. As regards a wrong choice of bacteria, we have started isolations of bacteria from the material of the experiments, in which leached cells are broken down in lake water.

Our experiments partly resemble those of WAKSMANN (1937), partly those of VON BRAND *et al.* (1937–1942).

Waksmann killed diatoms by suspending them in seawater in the dark, and concluded from the appearance of bacterial growth, that nitrogen was liberated from the diatoms.

Von Brand felt justified to conclude from his experiments, that the nitrogen from phytoplankton is converted in seawater quantitatively to ammonia, subsequently to nitrite and finally to nitrate. His experiments provoke criticism. Sometimes considerably more $N^{water}_{soluble}$ was recovered, than had been added as N^{cell}_{tot}. Von Brand assumed, that this increase came from N^{water}_{bound}, but that is impossible, as it appeared from his later experiments, that N^{water}_{bound} also increased after the addition of the plankton. Moreover, it can not be decided from his experiments whether the $N_{particulate}$ is still in the plankton or already in the bacteria. Finally it is remarkable, that such an intensive production of nitrite and nitrate can occur in seawater, while in fresh water without doubt it would have been inhibited considerably, when so much organic material had been added. It would therefore be important to know, to which extent the organic material in his experiments had been broken down.

The mineralisation of Si is a non-enzymatic, slow process. In our experiments only 20–30 % of the Si-compounds present in *Stephanodiscus Hantzschii*, was liberated after some weeks. Here the solubility of the formed product plays an important role, as appears from the effect of the addition of $NaHCO_3$. The percentage of Si-compounds liberated can vary considerably even in this single species.

Our experiments partly resemble those of Jørgensen (1955) with *Nitzschia linearis* and *Thalassiosira nana*. He found the liberation of the Si-compounds to be dependent on the pH. He used mixtures of $KHCO_3$ and K_2CO_3 as buffers. Whether his results were influenced by other factors than the pH's of his buffers was not investigated.

A distinct difference between both diatoms appeared: for *Nitzschia* at pH = 10 a maximal amount of 20 % of dissolved SiO_3'' was found after 85 days, whereas at the same pH for *Thalassiosira* this amounted to 97 %, after 37 days. As, moreover, in experiments with *Nitzschia*, all with the same pH, the rates of mineralisation varied considerably, he concluded, that various Si-compounds occur, with different hydrolysis rates.

The observation of Lewin (1958) that *Navicula pelliculosa* contains "stored" silicium, which might be chemically different from the "functional" silicium, points in the same direction. Little is known as yet about these compounds. It seems, however, probable, that the supposition of Engel (1953), that silicium is linked as silicic acid to galactose in rye straw, applies to diatoms as well. We intend to investigate whether, also from diatoms, two fractions can be isolated with a different silicium-galactose ratio, like Engel reported.

Cooper (1951) supposed the mineralisation of Si-compounds could be judged by means of microscopic examination of the cells. He concluded from Hart's communication (1934), that some diatoms could be recognised in the gastric contents of the animals that feed on these organisms, whereas others could not, that the Si-compounds must have been liberated from the latter cells. We fear his conclusion might be wrong, because *Thalassiosira* remains identifiable after digestion (Hart), while it has been shown in the experiments of Jørgensen, that the mineralisation of Si-compounds from this organism can be considerable. Moreover, the observation of Engel (1953) must be taken into account, that in rye straw the structure of the Si-deposits is still clearly visible microscopically, when 82 % of the silicium has been removed by extraction with methanolic NaOH.

When *Scenedesmus* cells were suspended in water during autolysis, iron could not be found outside the cells. When, however, EDTA was added to the suspensions 50–65 % of the iron appeared in the medium.

The effect of EDTA can be explained by assuming either that it extracts the – usually not liberated – iron from the cells "actively", or that it forms a stable complex with the liberated iron, which is otherwise precipitated immediately after liberation as alkaline iron-phosphate. The formation of such a precipitate (Einsele 1938) is certainly not improbable during autolysis, considering the high pH of the suspensions and the large amounts of phosphate liberated.

This precipitation might also be prevented by causing autolysis under anaerobic circumstances. In that case the iron might be liberated as ferrocompounds, which are better soluble. Mortimer (1941 and 1942) found that in lake water iron could dissolve under

anaerobic conditions as ferrocompounds; when afterwards conditions had become aerobic, these compounds were converted to ferri-compounds, which were precipitated.

Whatever the function of the EDTA may be, it is clear, that, in case of autolysis in a lake, the "chelate-forming" capacity of the water, which depends probably on the humus content, is an ecologically important factor.

From an ecological point of view the results of our experiments can be summarized as follows:

When an alga is killed under sterile conditions, 70–80 % of the P-compounds leave the cell in a few days, 70–80 % of the N-compounds remain in the cell, silicium leaves the cell only for a small part and very slowly, and iron is only dissolved when a stable complex can be formed, e.g. with EDTA.

The process of the liberation of N- and P-compounds was similar for all algae investigated. Therefore we feel justified to consider our results important for the interpretation of the phenomena observed in the development of algae in lakes, where no nutrients are supplied from the outside. Apart from the amount of autolysing cells in these lakes, the quite different liberation rates of the various elements will determine the growth rate of the algae.

The question now arises, whether the rates of liberation and mineralisation, observed during autolysis, have to be considered when we investigate the breakdown processes in a lake, were bacterial putrefaction always occurs.

In this connection we wish to discuss the experiments, already mentioned, of Kleerekoper (1952*a*, 1952*b* and 1953) more in detail. He concluded from analysis of living plankton and dead plankton collected in a lake at a depth of 11 m, that the mineralisation of nitrogen takes place more rapidly than that of phosphorus, as the phosphorus content of the dead material was very high. In our experiments on autolysis under sterile conditions the rates were reversed. As we found in preliminary experiments, that at least an important part of the N-slag, remaining after autolysis, can be digested by bacteria very rapidly, the experiments of Kleerekoper, though carried out under different circumstances, give at first the impression, that autolysis does not play an important role in the lake, studied by him.

In a later paper (Cooper e.a. 1953) it is suggested that the high P-content might be caused by a precipitation of alkaline ferriphosphate on the dead plankton. The occurrence of such a precipitate in a lake had also been reported already by Einsele (1936, 1938). The argument against autolysis in Kleerekopers experiments is refuted by this supposition, so that no conclusions can be drawn from this type of experiments as far as the ratio between the rates of autolysis and bacterial putrefaction are concerned.

This ratio, as found in laboratory experiments, may be used only

with the greatest reserve when judging similar phenomena in a lake, because bacterial breakdown in the laboratory will mostly be achieved with far higher concentrations of bacteria than in a lake.

We have tried in another way to acquire some understanding of the processes, occurring during the breakdown of cells in natural surroundings.

Algal growth was studied in concrete tanks with a capacity of 1–2 m^3, filled with lake water which had been filtered through plankton gauze. The concentrations of nitrogen, silicium and phosphorus were increased roughly to those of Rodhe's culture solution No. 8, to cause a sufficient bloom for quantitative chemical determinations.

Because of the bad summers of 1954 and 1956 no bloom of algae was obtained, but in 1955 a distinct growth was observed. Table VIII gives a survey of the variations in the concentrations of nitrogen, phosphorus and silicium and in the pH.

TABLE VIII. *Results of chemical analyses of an experimental pond during algal growth under semi-natural conditions.*

On the 2nd of June 1955 a concrete tank of 1.5 m^3 was filled with bog-water, filtered through plankton gauze (200 mesh). The inside of the tank was painted with Neodon-glas "S" (Neodon-Lackfabrik Helmut Sallinger Krumbach/Schwaben), to prevent dissolution of concrete into the water. The tank was dug into the ground and protected against the entrance of material by a glass roof, while gaps covered with nylon gauze prevented heating. — On the 4th of June quantities of NH_4NO_3, KH_2PO_4 and Na_2SiO_3 were added, till the concentrations mentioned below were reached. — In June a bloom of algae occurred; in July the algae died off. — The temperature and light intensity were nearly equal to those in a lake; they were registered, but are not given in the table. Evaporated water was replaced by distilled water. — Every morning the water was stirred. — The concentrations of the elements are expressed in mg/l.

	June 4	June 23	July 11	July 26	August 26
$P(PO_4''')$	1.97	1.23	1.08	1.36	1.13
P^{water}_{bound}	0.03	—	0.33	0.42	0.56
P^{cell}_{tot}	0.00	—	0.67	0.39	0.29
P^{susp}_{tot}	2.00	—	2.08	2.17	1.98
$N(NO_2')$	0.02	3.76	2.08	0.24	0.00
$N(NO_3')$	5.18	1.19	0.47	1.11	0.54
$N(NH_4^+)$	7.00	1.28	0.28	0.00	0.95
Sum	12.2	6.23	2.83	1.35	1.49
N^{water}_{bound}	3.4	3.74	5.47	4.74	4.00
N^{cell}_{tot}	0.00	—	4.35	3.76	2.66
N^{susp}_{tot}	15.6	—	12.7	9.9	8.15
$Si(SiO_3'')$	1.78	0.54	0.34	0.67	1.21
pH	7.8	8.2	8.4	8.1	8.2

As we are discussing the liberation processes during autolysis, affected by bacterial activity, we will only consider the breakdown period (July 11th–August 26th).

During this period the dissolved SiO_3'' increases. In the preceding period 1.44 mg of silicium had been incorporated, 60 % of which is liberated during the breakdown period. Therefore about 70 % of the silicium originally present, is again available for the growth of diatoms. Both Jørgensen's and our laboratory experiments yielded far lower values, which is remarkable, because the pH, although rather high for bog-water, is much lower than that, at which Jørgensen observed only a Si-liberation of a few per cent, even from *Thalassiosira.*

In our tank experiments the conversions of P_{tot}^{cell} and N_{tot}^{cell} come about more slowly than might be expected.

The N-balance shows a considerable deficit, which without doubt must be ascribed to the release of ammonia at these high pH's. Hence the nitrogen liberated during breakdown can not be recovered.

Nitrite was formed from nitrate, as appeared from experiments where KNO_3 was added to the lake water instead of NH_4NO_3. This phenomenon could be reproduced excellently. It is not plausible, that the reduction of nitrate stops at the level of nitrite; therefore a possible release of other gaseous reduction products than ammonia must be taken into account.

The experiments here described have a too preliminary character to determine exactly the ratio between autolysis and bacterial putrefaction, because the samples were drawn at too long intervals.

The impression is warranted, however, that the liberation of the P-compounds takes place more rapidly than that of the N-compounds, which suggests that autolysis can indeed occur in a lake under certain circumstances.

Before long we hope to repeat these experiments with some modifications, in order to establish a biocoenosis, which is an intermediate form between laboratory experiments and a natural lake.

SUMMARY

This paper describes an investigation into the function of the breakdown of algae in the cycle of elements in a lake.

The liberation and mineralisation of phosphorus, nitrogen, silicium and iron during sterile autolysis of *Scenedesmus quadricauda* and some other algae were determined. Autolysis was induced by chloroform treatment, U. V. irradiation or heating to 60° C.

70–80 % of the P-compounds are liberated in a few days, only the phosphate of the nucleic acids and proteins not being liberated. The forms in which phosphorus is liberated, and the mechanisms involved, were followed up. Some compounds are liberated by enzymatic activity.

Only 20–30 % of the N-compounds are liberated. The rest of the N-compounds belongs to the proteins and the nucleic acids, and remains as a slag.

The liberation of P- and N-compounds does not depend on size or nature of the killed algae investigated.

The liberation of Si-compounds is a non-enzymatic slow process. In 5 weeks 20–30 % of the Si-compounds were liberated from *Stephanodiscus Hantzschii*. This amount could be increased by adding $NaHCO_3$ to the suspensions of autolysing diatoms.

The mineralisation of iron, which is also a slow process, depends on the chelate-forming capacity of the milieu.

Preliminary experiments were carried out to digest the slag of sterile autolysis by means of bacteria. When the slag was suspended in lake water one half of the nitrogen was converted to ammonia in 5 days, while the other half was divided up into bacterial and *Scenedesmus* nitrogen, the mutual ratio of which could not yet be determined.

It appeared not yet to be possible to digest the slag by cultures of *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Bacillus subtilis*. The fact, however, that *Ps. fluorescens* and *Ps. aeruginosa* showed an increased respiration, when the slag was given as a substrate, and could also be cultured on a lye extract of the slag, is a stimulus for further investigations in this direction.

The combination of high light intensity and high temperature as a cause of the dying off of algae in nature is suggested to be a more frequently occurring phenomenon than is generally believed.

A preliminary experiment is mentioned, which gave probably a positive answer to the question whether autolysis can play a part in a lake, where bacterial putrefaction always occurs.

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THE INFLUENCE OF ANTAGONISTIC FUNGI ON THIELAVIOPSIS BASICOLA (BERK. ET BR.) FERRARIS

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CONTENTS

1. INTRODUCTION

Although *Thielaviopsis basicola* (Berk. et Br.) Ferraris can not be regarded as a severe parasite, the root-rot which it calls forth, may nevertheless cause considerable damage to certain host plants, in the field as well as in greenhouses. The fungus appears to be distributed in various kinds of soil, and almost all growers of root-rot susceptible plants become at one time or another confronted with it. Nevertheless, the presence of this root-parasite in the soil does not necessarily imply that susceptible plants will be severely attacked; in the same soil and with the same plant species the degree of injury may differ from year to year.

The variability in the pathogenicity of *Thielaviopsis basicola* has usually been ascribed to the influence exercised by physical factors either on the parasite itself or on the host plant. Conditions that are unfavourable to the development of the host, may give the fungus a better opportunity of attack, whereas conditions that are unfavourable to the fungus, may decrease its ability to penetrate into the tissues of a susceptible plant. A non-physical factor unfavourable to the development of *Thielaviopsis* in the soil might be the presence of micro-organisms that would be able to act as antagonists. It is rather striking that the influence of such organisms on *Thielaviopsis*, and indirectly on the disease caused by the latter, has so far received little or no attention. For this reason it looked attractive to study the influence which such micro-organisms, and especially some antagonistic fungi, may exercise on *Thielaviopsis*, in vitro as well as in the soil. In addition in two different soils the relation between the natural microflora and the population of *Thielaviopsis* was investigated during a period in which the soil was infested with the latter at regular intervals.

Out of a great many fungi that were isolated from different soils, a certain number proved to be antagonistic against *Thielaviopsis*, and among the latter some were selected for further study. Their antagonistic activity was measured by the aid of the effect which was exercised on the growth of *Thielaviopsis* by filtrates of the media in which they had been grown. The effect of external conditions, such as the nature of the medium in which the antagonists were cultivated, was also investigated. The effect of the antagonists on the pathogenicity of *Thielaviopsis* in the soil could be determined by estimating the severity of the disease symptoms shown by a sui-

table host. For this purpose *Nicotiana glutinosa* L. was chosen, because it is very susceptible to infection by this root parasite.

As far as is known to the author, no experiments have hitherto been carried out in this context with *Nicotiana glutinosa*. This plant was mentioned for the first time as a host of *Thielaviopsis* by JOHNSON (1916). When grown in soils that were heavily infested with tobacco black root-rot, its susceptibility appeared to be high. That the infection with *Thielaviopsis* has received no further attention, probably finds its explanation in the fact that this host was not considered to be of much, if any, economic importance. However, during the past few years it has become an important test object in the study of tobacco-mosaic virus.

As *Nicotiana glutinosa* appeared to be very sensitive to external conditions such as light intensity and temperature, the effect of a variation in these conditions on its development had to be known before an assessment of the pathogenicity of *Thielaviopsis* could be undertaken. This was the more necessary as adverse conditions of growth may cause symptoms which are very similar to those caused by the infection. It might also be that plants which did not show any symptoms of decline caused by physical factors, had decreased in their sensitivity to *Thielaviopsis*. In order to test this possibility the plants that were exposed to different combinations of external conditions, were grown partly in a soil that was infested with *Thielaviopsis*, and partly in a soil that was not infested. For further experiments that combination of conditions was chosen which had proved to be as favourable as possible for a good growth of the plants and which still allowed an accurate assessment of the severity of the disease symptoms caused by *Thielaviopsis basicola*.

2. SOME REMARKS ON THE HISTORY OF THE SUBJECT

2. 1. On THIELAVIOPSIS BASICOLA AS CAUSE OF THE "BLACK ROOT-ROT" AND ON VARIOUS FACTORS AFFECTING THIS DISEASE

A cursory survey of the literature dealing with *Thielaviopsis basicola* (Berk. et Br.) Ferraris and with the "black root-rot" which is caused by this fungus, will suffice to convince us that this parasite, which was described for the first time in 1850 by BERKELEY and BROOME under the name *Torula basicola*, has already drawn considerable attention.

In the earlier publications there is often some confusion with regard to its identity, because this fungus was regarded for some time as the conidial stage of an ascomycete described in 1876 by ZOPF as *Thielavia basicola*. On account of this mistaken identification the fungus of BERKELEY and BROOME received in 1912 from FERRARIS the name *Thielaviopsis basicola*. However, in 1925 MCCORMICK could prove that the fungus of which the perithecial stage had been described by Zopf as *Thielavia basicola*, is a distinct species, and that the association with the imperfect fungus *Thielaviopsis basicola* is but accidental. Further evidence in support of this view was provided by LUCAS (1948) and by HÄRRI (1959).

Thielaviopsis basicola is able to infect a large range of plants. Already in 1916 more than a hundred species belonging to 18 families were, according to JOHNSON, known as hosts, and since then their number has still further increased. GARRETT (1956) regards *Thielaviopsis basicola* as a primitive, unspecialized parasite, and he adds that although strains of different virulence occur, they all seem to agree in the wide range of their hosts. With regard to the damage caused by this fungus it may be remarked that it is really destructive only to young plants and to plants growing under somewhat adverse conditions.

Thielaviopsis basicola occurs in different soils. YARWOOD (1946) collected in 12 Californian localities 17 soil samples, and succeeded in isolating *Thielaviopsis basicola* from 12 of them; the latter were obtained from 7 of the 12 localities. He further found that the fungus was present in soils where crops were grown which showed no symptoms of the disease. STOVER (1950b) reported that *Thielaviopsis* was prevalent in several fields that were situated over 100 miles from the old tobacco areas, and on which as yet no more than three tobacco crops had been grown. For this reason he assumes that this fungus must be able to live in soils on which no susceptible plants occur. Under such circumstances it must be able to persist partly as a weak parasite and partly as a saprophyte until the introduction of a highly susceptible host offers it the opportunity to develop its full parasitic vigour. The possibility of a survival in susceptible weeds is not considered by him. An inquiry instituted in the Netherlands by MOOI–BOK (1952) revealed that the fungus has been found here nearly everywhere where *Lathyrus odoratus* is grown.

Special studies have revealed that the effect exercised by *Thielaviopsis basicola* on susceptible plants is variable, and that this variability depends on external conditions. The influence of the latter on the black root-rot of *Nicotiana tabacum* has been thoroughly investigated, but little or no research has been carried out so far on the black root-rot of *Nicotiana glutinosa*, the plant that was used as host in most of our own investigations. However, a brief survey of the studies that have been carried out with *Nicotiana tabacum* as host, will be of interest.

From the work of JOHNSON and HARTMAN (1919) on the influence of the soil factors it is clear that in tobacco the severity of the black root-rot is determined in the first place by the temperature of the soil. Relatively low temperatures favour the development of the disease, but higher temperatures are less suitable for it; above 26° C the infection rapidly declines, and at about 30° C hardly any trace of the infection is left. Similar results were obtained by VALLEAU, KENNEY and KINNEY (1925). They report that at soil temperatures between 21° C and 23° C black root-rot is a serious menace to tobacco, but that at an average soil temperature of 25.5° C the crop suffered hardly at all. The findings of DORAN (1929) and of JEWETT (1938) point in the same direction. The latter found that at temperatures between 18° C and 20° C the number of tobacco varieties that showed symptoms of the disease, was larger, and that the infection was much

more severe than it appeared to be at temperatures between 28° C and 30° C. According to STOVER (1950b), the range of temperature which is most suitable for the estimation of the pathogenicity of *Thielaviopsis basicola*, would be found between 18° C and 25° C.

According to CONANT (1927) the differences in resistance to the attacks of *Thielaviopsis basicola* that are shown by tobacco at various temperatures, would be due to differences in its capacity to produce a layer of cork in the tissue underlying the lesion. JEWETT (1938), however, could not confirm these findings of Conant.

Infection experiments (GILBERT, 1909; JOHNSON and HARTMAN, 1919; LUCAS, 1955, and others) showed that the most favourable temperature range for the infection is 17° C to 23° C, and that it does not correspond therefore with the optimum temperature found for the growth of the fungus in pure cultures nor with the optimum temperature for the growth of the host, in both cases 25°–30° C.

Investigators generally agree that a pH exceeding a value of approximately 5.6 is favourable to the development of black root-rot in tobacco. BRIGGS (1908) concluded from his experiments that substances which render the soil more alkaline, cause an increase of the black root-rot, whereas addition of substances with an acidifying effect cause a decrease of the disease. The results obtained by JOHNSON and HARTMAN (1919) with pot experiments point in the same direction. In the field experiments carried out by ANDERSON, OSMUN and DORAN (1926) it was found that black root-rot caused little or no damage to the tobacco crop when the pH of the soil remained below 5.6, whereas the damage became severe when the pH of the soil exceeded 5.9. The critical range, therefore, lies between pH 5.6 and pH 5.9. The position of this critical range is not fully constant, but may shift somewhat under the influence of the temperature and of other factors. They studied the influence of the pH also in pure cultures of *Thielaviopsis basicola*; the results obtained with the latter proved to be in agreement with those arrived at in the field experiments. The results obtained by these investigators were confirmed by ANDERSON and MORGAN (1926) and by MORGAN and ANDERSON (1927).

DORAN (1929) found that there was little or no black root-rot so long as the pH of the soil remained below 5.6, no matter what the temperature was. However, the point at which the pH began to allow the development of the disease, appeared to be markedly influenced by the temperature. At 15° C the disease made its appearance at a pH of 5.7, at 18° C at a pH of 5.7–5.8, at 21° C–24° C at a pH of 5.8, at 27° C at a pH of 5.8–5.9. At a temperature of 30° C there was hardly any injury at all, even when the pH reached values ranging from 6.0 to 6.9. The critical pH-value, therefore, appeared to increase with the temperature. In a later report (1931) he confirmed the findings of Briggs by showing that the infection by *Thielaviopsis* decreased when the pH of a limed soil was subsequently lowered by acidification.

Next to the temperature and the pH, the moisture content of the

soil is of importance. GILBERT (1909) concluded from his experiments that excessive watering is favourable to the development of black root-rot in tobacco. JOHNSON and HARTMAN (1919) confirmed his findings, and gave his conclusion a somewhat more precise form by stating that the soil humidity is of importance only when the soil is very wet or when it is saturated with water; under such circumstances infection by *Thielaviopsis basicola* is more severe. With regard to the humidity of the air no experiments have as yet been performed.

JOHNSON and HARTMAN (1919) and ANDERSON, OSMUN and DORAN (1926) studied the relation between the degree of black root-rot of the tobacco crop and the amount of *Thielaviopsis* inoculum that is present in the soil. They agree with each other that the degree of damage is directly proportional to the number of places at which the roots become infected. In order that the disease may assume a serious form, it is therefore necessary that the soil contains a high amount of inoculum. More precise data were supplied by LEVYKH (1938). He studied the problem by means of pot experiments, and found that tobacco seedlings proved to be but very slightly infected and that the number of them that could be transplanted, was but slightly reduced when the soil was infested with less than 100 chlamydospores per cc soil; that adult tobacco plants were slightly infected when the soil was infested with 100–1000 chlamydospores per cc; that a severe infection was obtained when the number of chlamydospores was raised to 3.312 per cc, and that with 5.780 chlamydospores per cc the highest degree of infection was reached. STOVER (1950b) too found that the effect of an infestation is markedly influenced by the amount of inoculum. If the latter is either very small or very high, it is impossible to determine the pathogenicity of the strain that is used for the infestation, from the effect it produces; if the amount exceeds a certain level, the effect remains the same, and if the amount remains below a certain level, there is no effect at all.

The authors whose publications were reviewed above, have shown that the pathogenicity of *Thielaviopsis basicola* may be influenced by several factors, soil temperature, pH of the soil, quantity of inoculum that is available, character and condition of the host, etc.

However, there is one factor that has been left out of consideration by previous investigators, and of whose influence on *Thielaviopsis basicola* as yet little is known. This is the presence of antagonistic micro-organisms. As a background for the investigations to be carried out on the influence exercised by such organisms on *Thielaviopsis basicola*, it seems appropriate to consider some general aspects of microbiological antagonism.

2.2. ANTAGONISM BETWEEN MICRO-ORGANISMS AND THE SIGNIFICANCE OF THIS PHENOMENON TO PHYTOPATHOLOGY AND SOIL MICROBIOLOGY

The existence of an antagonism between various micro-organisms was known already towards the end of the preceding century, but it was especially during the last decades that it became an object of

more general interest to microbiologists as well as to phytopathologists and medical men. Several handbooks dealing with this topic have already been published, e.g. WAKSMAN (1945), HERRELL (1945), FLOREY *et al.* (1949), VOGEL (1951). Several times too the subject has been reviewed in journals, e.g. by WAKSMAN (1937), WEINDLING (1946), BRIAN (1951), WOOD and TVEIT (1955). The number of special papers has increased to such an extent that in this short survey but a small part of them can be mentioned.

2.2.1. *Antagonistic micro-organisms and their products*

POTTER (1908) found that *Pseudomonas destructans* Potter (= *Erwinia carotovora* (Jones) Holland), the cause of the "turnip rot", produced a substance by which this organism itself may be killed. This substance proved to be heat-resistent and more or less specific for the organism by which it is produced. It appeared, moreover, that the "turnip rot" could be combated by treating the turnips with this toxin. Potter therefore was one of the first to make use of microbial antagonism in the control of a plant disease. In a similar way it appeared possible to control *Penicillium italicum* Wehmer, which causes a disease in oranges, by means of a substance produced by this fungus in its own metabolism. Potter supposes that the faculty to produce such substances may be more generally distributed, and that it may provide us with a means to control some of the plant diseases.

Another important contribution to our knowledge of microbial antagonism was made by PORTER (1924). The latter found that the growth of *Helminthosporium* may be inhibited by various kinds of bacteria, and he could show that one of these bacteria produced a diffusible substance which inhibited the growth of *Helminthosporium* already at a distance of 2 cm from the place where it was produced. A distinct protection of wheat and flax seedlings against infection by *Helminthosporium* and *Fusarium* respectively was obtained by the use of such antagonistic bacteria.

BAMBERG (1930) reported that he had succeeded in isolating from maize plants a bacterium which inhibited the infection of these plants by *Ustilago zeae* (Beckm.) Unger and which destroyed the galls caused by this fungus when the latter had already been developed.

That micro-organisms which are normally present in the soil, may exercise an inhibiting effect on a soil-borne disease, was recognized by HARTLEY (1921). This author carried out infection experiments with *Pythium debaryanum* Hesse, and found that the conifer seedlings used for these experiments showed much more "damping off" when the soil in which they were grown, was previously sterilized, than when it was left unsterilized and that the disease could, to a large extent, be kept in check by an infestation of the soil with saprophytes like *Rhizopus nigricans* Ehrenb.

HENRY (1931) made a similar observation with regard to the pathogenicity of *Helminthosporium sativum* Pk., the cause of "foot-rot" in wheat. This parasite may be kept in check by means of various soil microbes; the more saprophytic organisms were added to the

sterilized soil, the greater the antagonistic effect on *Helminthosporium* appeared to be.

Tims (1932) could show that various actinomycetes which were isolated by him from sugar-cane soils, exercise a strong antagonistic effect on a *Pythium* that grows as a parasite on sugar-cane. In infection experiments that were carried out in sterilized soil with sugar-cane as well as with wheat, the root-rot of these plants could be reduced by inoculating the soil with a culture of one of the more active antagonists.

In test-tube experiments with cabbage seedlings grown from sterilized seeds, Jaarsveld (1942) found that various soil fungi are able to reduce the pathogenicity of *Rhizoctonia solani* Kühn. All soil fungi that were tested in these experiments, proved to exercise an antagonistic action, and when more than one of these fungi were added, the effect proved to be increased. Experiments with potted plants led to more or less similar results.

A noteworthy contribution to our knowledge was brought by the paper of Van Luyk (1938). He found that various fungi may exercise an antagonistic action on species belonging to the genus *Pythium*; and the effect which culture filtrates of *Pullularia pullulans* (de Bary) Berk. and of *Penicillium expansum* (Link) Thom exercise on the growth of *Pythium debaryanum*, was estimated quantitatively in pure cultures of the latter. Remarkably effective were the filtrates obtained from *Penicillium expansum;* even in a dilution of 1 to 1280 they inhibited the growth of the parasite. Van Luyk's greenhouse experiments with grass and lucerne showed that the infection of these plants by *Pythium debaryanum* was strongly reduced by the activity of the antagonists. He also studied the way in which the antagonistic activity of the filtrate of *Penicillium expansum* is influenced by the carbon source present in the culture medium, and investigated some of the properties shown by the metabolic products of this antagonist. The antibiotic "expansin" that is involved here, was isolated by Oosterhuis in 1946.

Slagg and Fellows (1947) tested the effect of 143 different kinds of soil fungi on *Ophiobolus graminis* Sacc., and found that about one quarter of them exercised an antagonistic action. In pure cultures these antagonists formed "by-products" which proved to inhibit the growth of *Ophiobolus graminis*, and in artificially as well as in naturally infested soil several of them were found to decrease the damage caused by this parasite to wheat. Similar instances of antagonism between soil fungi and soil-borne plant parasites have repeatedly been reported in subsequent years (Cooper and Chilton, 1950; Luke, 1952a, 1952b; Johnson, 1952, 1954; etc.).

A large part of the investigations on the antagonism between micro-organisms have been carried out either in the laboratory by means of pure cultures or else in the greenhouse, where the host plants were grown in a sterilized and subsequently artificially infested soil. However, it is not a priori certain that antagonism will play a similar rôle in natural soils with their complex microbiological popu-

lation. GARRETT (1956), who in his handbook "Biology of root-infecting fungi" has summarized his own observations as well as those of other investigators, points out that the intricate composition of the microbial population in natural soils is to be regarded as one of the factors which tends to obscure the effect of the antagonism which is observed in pure cultures and in sterilized soil after an artificial infestation.

Several investigators have noted that the soil fungi belonging to the genus *Trichoderma* are of importance as producers of antibiotic substances. In 1932 WEINDLING described the way in which the hyphae of *Rhizoctonia solani* and of other fungi were parasitized by *Trichoderma lignorum* (Tode) Harz, and BISBY, JAMES and TIMONIN (1933) and BROWN (1933) observed the parasitism of this fungus on *Fusarium culmorum*, on *Helminthosporium sativum* and on *Phymatotrichum omnivorum*, and remarked that it might aid in controlling the phytopathogenic fungi living in the soil. Some other observers too have noted the antagonistic action of *Trichoderma*, e.g. BUTLER (1935), ALLEN and HAENSELER (1935) and DAINES (1937).

WEINDLING had noted already in 1932 that in a culture medium *Rhizoctonia solani* may be destroyed by a substance or by substances secreted by *Trichoderma lignorum*. In 1934 he published a paper dealing with the "lethal principle" produced by this fungus; some of the properties of the latter were described, and the author added that there is evidence that this lethal principle is a single chemical substance. Two years later WEINDLING and EMERSON (1936) announced the isolation of an antibiotic from the culture filtrate of *Trichoderma lignorum*, and described the chemical properties of this substance, which in later papers of WEINDLING (1937, 1941) is called "gliotoxin". The production of this antibiotic by *Trichoderma* was studied also by BRIAN (1944), BRIAN and HEMMING (1945) and others.

Subsequently BRIAN and MCGOWAN (1945) and BRIAN, CURTIS, HEMMING and MCGOWAN (1946) succeeded in isolating another antibiotic from strains of *Trichoderma viride* Pers. ex Fr. This substance, for which they proposed the name "viridin", is a stronger antibiotic than gliotoxin, but it is less stable. Afterwards BRIAN (1951) found that most strains of *Trichoderma viride* produce a mixture of gliotoxin and viridin.

2.2.2. *Production and rôle of antibiotics in the soil*

Earlier studies have led to the discovery of a number of antibiotics of which in 1951 BRIAN has given a summary. It appears from the latter that 2191 species of fungi belonging to 245 genera had already been tested, and that 785 species belonging to 120 genera had been found to produce active substances. Moreover, some 96 antibiotics had already been recognized, and 57 of the latter had been obtained in a pure form and are well characterized. The remainder were known only in crude extracts. In an earlier work BRIAN (1949) had suggested that "the capacity to produce antibiotics is particularly characteristic of micro-organisms whose natural habitat is in the soil,

and that this capacity may be in some cases a factor concerned in the maintenance or change of the microbiological balance in the soil, and thus indirectly concerned with soil fertility."

An extensive study on the production and rôle of antibiotics in the soil was started in 1951 by SIMINOFF and GOTTLIEB. One of their aims was to find out whether antibiotic substances are produced in the soil, and if so, whether they exert any influence on its microbiological population. This work was continued by GOTTLIEB and SIMINOFF (1952), MARTIN and GOTTLIEB (1952), GOTTLIEB, SIMINOFF and MARTIN (1952) and MARTIN and GOTTLIEB (1955). They studied the production in the soil of 11 antibiotics formed by well-known antagonists, and investigated the part played by these substances and their ultimate fate. They arrived at the conclusion that some of them are soon inactivated, for instance by adsorption to the soil particles. Some other ones, however, appeared to play a more active rôle in the life of the microbiological community.

JEFFERYS (1952) has studied the stability of 10 antibiotics in different soils. In contrast with the investigators quoted above, he added solutions of antibiotics to the soil. He found that the antibiotics differed from each other in their stability, and that the latter varied from soil to soil. Nevertheless, in some of the soils they all exhibited a fair degree of stability. Their inactivation rests in his opinion on four causes, viz. (1) an unfavourable pH of the soil, (2) adverse microbial activity, (3) their adsorption to soil particles, and (4) an as yet unknown factor, possibly of a chemical nature.

NISSEN (1954) studied the effect exercised by high concentrations of antibiotics on the complex micropopulation found in the soil. In order to obtain an idea of the changes in microbiological activity in the soil he estimated the production of carbon dioxide in the latter, as he regarded the production of this substance as a measure of the microbiological activity. He experimented with five antibiotics, and found that they differed in the rate at which they were inactivated in the soil.

The results obtained by JEFFERYS, BRIAN, HEMMING and LOWE (1953) seem to lend support to the view that the production of antibiotics is of ecological importance to the fungus flora of the soil. They found that 45 % of the wide-spread and locally abundant fungi of acid heath soils were able to produce these substances, whereas but 15 % of the rarer fungi were able to do so.

The findings of DOBBS and HINSON (1953) indicate that in natural soils a "fungistasis" is of common occurrence. This view is shared by JEFFERYS and HEMMING (1953), who are of opinion that this may find its explanation to some extent in the activity of the antibiotics. They add that their observations suggest the presence of discontinuous "islands" of inhibition rather than that of a wide-spread "sea" of inhibition. The presence of inhibitory material in the soil was confirmed by HESSAYON (1953), CHINN (1953), STOVER (1955, 1958) and others.

The way in which the inhibitory substances affect the spores and

hyphae of the fungi, has been studied too. Park (1955, 1956) observed a lysis of fungus spores in contact with soil particles. Jackson, who reported in 1958 on the presence of a fungistatic factor in Nigerian soils, tested the fungistatic effect of the soil on 19 species of fungi, and found that in 11 of them the germination of the spores was distinctly inhibited. Lockwood (1959) states that natural loam causes in various fungi a lysis of the mycelium, and inhibits the germination of the conidia. He ascribes this to the presence of lytic and toxic substances produced by species of *Streptomyces*, and he succeeded in demonstrating the presence of such substances in the soil.

The findings of the investigators quoted above indicate that fungitoxic substances are present in the soil, and that the latter doubtless play an important part in the maintenance of the microbiological balance in that habitat.

2.2.3. *Antagonism against Thielaviopsis basicola*

The results obtained in the study of microbial antagonism suggest that the differences in pathogenicity shown by *Thielaviopsis basicola* may to some extent be due to the activity of the antagonistic microorganisms by which it may be accompanied. However, so far but little was known of the influence which such organisms may exercise on this parasite. Tiddens (1933) found that *Primula obconica* is more severely infected by this fungus when it is grown in sterilized soil than when it roots in an unsterilized one. Stover (1950a) reports that in cultures of this root parasite the growth of the latter was sometimes inhibited by the presence of an unidentified bacterium. Mooi–Bok (1952) observed that in cultures of *Lathyrus odoratus* the infection by *Thielaviopsis basicola* decreased when *Fusarium oxysporum* Schl. was added to the soil. She also carried out experiments on the direct influence of "antagonists" on *Thielaviopsis basicola* in order to find out whether they might be used for a biological control of the latter, but the results were on the whole negative.

3. ON THE ISOLATION OF THIELAVIOPSIS BASICOLA AND ON THE SELECTION OF SUITABLE ANTAGONISTS

3.1. Isolation of thielaviopsis basicola

Diseased *Primula obconica* plants, infected by *Thielaviopsis basicola*, were examined macroscopically as well as microscopically. The disease symptoms caused by this parasite are readily recognizable; one is a yellow discoloration which comprises the whole leaf surface with the exception of a narrow zone along the principal veins; another symptom is to be found in the brown to black discoloration of the rotting roots. In the infected parts of the root system chlamydospores of the parasite proved to be present in large amounts. In order to isolate the pathogen, fragments of the diseased roots were superficially sterilized and laid out on cherry agar. In many instances not only *Thielaviopsis basicola*, but also species of *Pythium*, *Fusarium*

and *Cylindrocarpon* were found to grow out from the root fragments. However, it was not difficult to obtain *Thielaviopsis basicola* in pure culture.

When *Nicotiana glutinosa* was infected with *Thielaviopsis basicola*, the diseased roots soon turned brown, and in that case they showed numerous lesions ranging in colour from brown to black. Contrary to what happens in *Primula obconica*, not only the young roots appear to be affected but also the older ones which are often entirely covered by large black, slightly swollen patches. The taproot may show the symptoms of the disease right up to the base of the stem. The young roots are probably soon killed by the parasite, and for this reason it is more difficult to isolate *Thielaviopsis basicola* from *Nicotiana glutinosa* than from *Primula obconica*. The older roots nevertheless contain not only the dark-coloured mycelium of the parasite but also large numbers of chlamydospores. A successful method for isolating *Thielaviopsis basicola* consisted in rinsing the diseased roots in running tap-water for at least 15 hours, dipping them for a few seconds in 96 % alcohol, and subsequently washing them in sterilized water; then the roots were dried on sterilized filter-paper, divided in small parts, and laid out on cherry agar. When the cultures were 2–3 days old, spore suspensions were made of them, and with the latter pure cultures of the parasite could be obtained. In most instances not only *Thielaviopsis* but *Fusarium* too developed from the roots that had been laid out, and this was a great drawback, for it proved particularly difficult to separate *Thielaviopsis basicola* from this fungus. It does not seem impossible that *Fusarium* too may impart a disease to *Nicotiana glutinosa*.

A modification of the carrot-disc method introduced by YARWOOD (1946) has also been used for the isolation of *Thielaviopsis basicola* from diseased roots of *Nicotiana glutinosa* (STOVER, 1950a). The roots were thoroughly washed, macerated and spread out on the surface of fresh carrot discs. Within a few days greyish-green to almost black colonies of *Thielaviopsis basicola* appeared on the surface of the latter; the large masses of endoconidia that were formed by these colonies, could easily be transferred to Petri dishes or test tubes containing a suitable agar nutrient. This method proved to yield much better results than the method of superficial sterilization described above.

The method of YARWOOD (1946) has also been used for isolating *Thielaviopsis basicola* from soil. Fresh, approximately 0.5 cm thick carrot discs were placed in Petri dishes, and covered with a 0.5 cm thick layer of soil taken from pots in which diseased tobacco plants had been grown. Then a slight pressure was exercised on the soil, and an amount of water was added sufficient to moisten the soil, but leaving no surplus in the Petri dish. After the Petri dishes had been kept for about three days at room temperature, the carrot discs were washed in order to remove the soil, and placed in sterile moist chambers that were kept in an incubator at approximately 23° C. After about 24 hours in the moist chambers several grey colonies of *Thielaviopsis basicola* were already distinguishable on the

surfaces of the carrot discs Microscopic examination revealed that at this stage an extensive mycelium was present with a large number of conidiophores and of endoconidia, but as yet without chlamydospores. Pure cultures therefore could already be obtained. After a sojourn of 3 to 4 days in the incubator the carrot discs proved to be entirely overgrown with the greyish-green to nearly black fungus, and although conidiophores and endoconidia still prevailed, chlamydospores too were now present in a considerable amount.

Soil samples taken from various gardens at Baarn have been tested in this way, and it appeared that *Thielaviopsis basicola* is rather common in this place. The method proved to be particularly suitable because it requires but little time, and so it could, for instance, easily be found out whether *Thielaviopsis basicola* is present in a sufficient amount to cause black root-rot in tobacco plants.

3.2. Isolation of antagonistic fungi

The name antagonist will here be used for those fungi which exercise unmistakably an inhibiting influence on the development of *Thielaviopsis basicola*, or which are able to cause lysis of the mycelium.

In order to obtain antagonists, the fungus flora of clay and sand soils as well as of other likely substrates, e.g. leaf-mould, was examined. At a later stage also "diseased" and "healthy" *Primula* soils, some of which had been periodically infested with *Thielaviopsis basicola*, were tested as to the presence of antagonists. Two different methods were applied for obtaining the latter.

In the first place suspensions were prepared of 10 soils, and for the analysis of the fungus flora of each soil 10 Petri dishes were set apart. The suspensions were mixed with molten cherry agar at 40° C and the mixture poured out in the Petri dishes. Immediately after the plating-out, the mixture was inoculated at 5 different spots with *Thielaviopsis basicola*. The suspension should be diluted so far that in each Petri dish 10–30 fungus colonies will develop. The pH of the medium should lie between 4 and 5.

When the second method was followed, Petri dishes with cherry agar, were used, in the center of which *Thielaviopsis basicola* was inoculated. After being incubated for 5 days at 24° C, each colony of *Thielaviopsis* was surrounded at 5 places with soil particles.

The second method is certainly not an ideal one, but it nevertheless yielded good results. A distinct advantage is that it saves time and material, which is especially important when a large number of soils have to be tested. It may be made even more effective by inoculating each agar plate with more than one colony. The principal disadvantage of this method is that the development of some of the fungi that are present in the soil particles, may be made impossible by that of the more vigorously growing ones, so that they find no opportunity to display their antagonistic action.

By means of these two methods altogether 38 fungi were obtained which on cherry agar exercised an inhibiting effect on the growth of *Thielaviopsis basicola*. Of these 38 fungi pure cultures were made.

3.3. Selection of the most active antagonists

Among the fungi that had been isolated by means of the two methods described in the preceding section, those whose culture filtrates displayed the strongest antagonistic activity with regard to *Thielaviopsis basicola*, were selected for further study.

In order to obtain the culture filtrates, the fungi were cultivated in cherry juice (50 cc per Erlenmeyer flask with a capacity of 300 cc). They were incubated for 20 days at a temperature of 24° C, after which the culture liquid was passed through filter-paper and then through a sterilized Seitz filter.

The culture filtrates that had been obtained in this way, were diluted by means of cherry juice to one half, one quarter or one eighth of their original strength, and these dilutions were divided in amounts of 10 cc over test tubes; in the latter they were inoculated with *Thielaviopsis basicola*; test tubes filled with 10 cc cherry juice were also inoculated, and served as control. The test tubes were kept in an incubator at 24° C.

In the test tubes which contained cherry juice without any addition of culture filtrate, the development of *Thielaviopsis basicola* was good; the fungus covered the culture liquid with a thick layer of mycelium; after about 8 days growth came to a stop. In the other test tubes the development of the fungus was sometimes equal to that in the control tubes, but in other instances it proved to be inhibited to a more or less considerable degree. About 20 % of the fungi that were tested in this way, proved to be markedly antagonistic. This applies, of course, only to fungi that are grown on cherry juice; it is not impossible that other values will be found when the experiments are carried out with filtrates obtained from other culture media.

In subsequent experiments use was made only of those fungi whose culture filtrates had proved to be most strongly inhibiting. These fungi are:

Aspergillus fumigatus Fres., strain 1
Aspergillus fumigatus Fres., strain 2
Penicillium spiculisporum Lehman
Penicillium spinulosum (Link) Thom
Penicillium expansum (Link) Thom
Penicillium roqueforti Thom
Gliocladium roseum Bain.
A sterile mycelium

In order to obtain more active strains of these fungi, of each of them 5 mono-spore cultures were made, and the effect of their culture filtrates on *Thielaviopsis basicola* was compared in experiments performed under the same circumstances. Here too the filtrates were obtained from cultures in cherry juice in the way described above. It appeared that the filtrates of some of these mono-spore cultures exercised a considerably stronger inhibiting effect than others did that were derived from the same original culture. The most active strains were kept in culture in order to serve in subsequent experiments.

4. ON THE ANTIBIOTIC ACTIVITY OF THE SELECTED ANTAGONISTS

The experiments described in this chapter were carried out in order to determine the influence exercised by various external factors on the production of substances which inhibit the development of *Thielaviopsis basicola.* In addition, the production of these substances by various strains of *Penicillium roqueforti* was compared, and also the effect of the culture filtrate of this fungus on different strains of *Thielaviopsis basicola.*

4.1. Survey of the literature dealing with the antibiotics produced by the above mentioned antagonists

That *Aspergillus fumigatus* may exercise an antagonistic influence, was demonstrated already in 1913 by Vaudremer (quoted from Waksman, 1945). Since then several antibiotics have been isolated from the culture filtrates of this fungus, viz. fumigatin (Oxford and Raistrick, 1942; and others), spinulosin (Oxford and Raistrick, 1942; and others), fumigacin, which proved to be identical with helvellic acid (Waksman, Horning and Spencer, 1943; Menzel, Wintersteiner and Hoogerheide, 1944; Waksman and Geiger, 1944) and gliotoxin (Glister and Williams, 1944; Menzel, Wintersteiner and Hoogerheide, 1944).

Of *Penicillium spinulosum* it is known that it produces spinulosin, a substance isolated and studied by Birkinshaw and Raistrick (1931). Nothing has so far been reported on an antagonistic action of this *Penicillium* on other fungi.

Penicillium expansum has already repeatedly been mentioned as an antagonist of phytopathogenic fungi. Van Luyk (1938) studied its antagonism with regard to *Pythium debaryanum* in vitro as well as in vivo, and discovered that it produces a particularly active culture filtrate. Jaarsveld (1942) used it as an antagonist for the control of *Rhizoctonia solani.* The antibiotic was isolated from the culture filtrate in 1946 by Oosterhuis, who called it expansin.

Little is known of the antagonistic activity of *Gliocladium roseum* itself, but another species of the genus *Gliocladium*, viz. *Gliocladium fimbriatum* Gilm. et Abbott, is known to produce gliotoxin (Weindling, 1937, 1941) and to exercise an antagonistic influence on other fungi.

Of an antagonistic activity of *Penicillium roqueforti* and of *Penicillium spiculisporum* nothing was known so far. In the tables published by Florey *et al.* (1949) these two fungi are mentioned among those that have been tested with regard to their antibiotic activity with negative result. By Jefferys, Brian, Hemming and Lowe (1953), who studied the antibiotic activity of 65 fungus species isolated from acid heath soils, *Penicillium roqueforti* was tested in two different ways, but it was found to be inactive with regard to fungi as well as with regard to bacteria.

In view of the fact that so far nothing was known of an antibiotic activity of *Penicillium roqueforti* and of *Penicillium spiculisporum*, special attention was paid to these two fungi.

4.2. Production of antibiotics in organic media

In the experiments described above the antagonists were cultivated in a cherry-juice medium. However, as the composition of the medium in which they are cultivated, may influence the antagonistic activity of the culture filtrate, another series of experiments was carried out in which the same antagonists were cultivated in another medium, viz. in a potato extract.

In these experiments young and vigorously growing mycelium of each of the antagonists was transferred to a series of 7 Erlenmeyer flasks with a capacity of 300 cc. Each of the latter contained 50 cc of the sterilized cherry or potato extract. It was hoped that in this way of each antagonist at least 200 cc culture filtrate would become available. The culture flasks were kept in an incubator at 24° C for a period of 16 days; at the end of this period the culture liquid was filtered, and the filtrate diluted respectively to one half, one quarter, one eighth, one sixteenth and one thirty-second; part of it was used without diluting it.

Of each of the undiluted and of the variously diluted fractions of the filtrate, 5 samples of 20 cc were poured in Erlenmeyer flasks with a capacity of 100 cc. A control series consisted of 15 similar flasks filled with 20 cc of the original culture medium. For the inoculation were used round discs 3 mm in diameter taken from 10 days old cultures of *Thielaviopsis basicola*; the latter had been isolated from *Primula obconica*, and was cultivated on cherry agar. After the inoculation, the flasks were placed in an incubator with a temperature of 24° C, in which they were kept for a period of 14 days.

As a measure for the antagonistic activity of the filtrate it was decided to use the dry-weight of the mycelium. This was determined a short time before the controls had reached their maximum development, as with a longer period of growth the differences between the uninhibited and the variously inhibited mycelia would have been obscured. First of all, therefore, some experiments were performed in order to determine the length of the period which an uninhibited *Thielaviopsis* culture requires to reach its maximum development.

The contents of the flasks were filtered and the residuum washed and sucked dry; then it was dried for an hour at 70° C, and subsequently for 3 hours at 103° C; after cooling, the filters with the mycelium were weighed, and from the gross weight the previously determined weight of the filter subtracted. This does not yet give the dry-weight of the amount of mycelium produced during the period of growth; it still includes the dry-weight of the material that was used for the inoculation, and that of the particles of dust and of other insoluble material that may have been present in the filtrate and in the culture medium. In order to determine the weight of these contaminations the contents of a number of flasks in which no growth had taken place, was filtered, and the residuum treated in the same way as that obtained from the other flasks. By averaging the values found in this way, the dry-weight of the inoculation material and of insoluble particles derived from other sources was found, and so

the nett weight of the mycelium produced during the period of cultivation could be determined.

The results of experiments in which the antagonists had been cultivated in cherry juice, are given in Table 1.

TABLE 1

Dry-weight in mg of the mycelium of *Thielaviopsis basicola* developed in undiluted and diluted filtrates of the cherry-juice medium in which one of the listed antagonists had been cultivated

Antagonists	Degree of dilution of the filtrate					
	1	1/2	1/4	1/8	1/16	1/32
A. fumigatus I	0	0	0	0	0	50.0
A. fumigatus II	0	0	0	0	43.0	151.9
P. spiculisporum	0	0	0	22.7	112.5	185.2
P. spinulosum	0	0	0	87.5	161.5	223.7
P. expansum	0	0	0	0	0	0
G. roseum	0	142.4	239.5	245.0	266.8	272.0
P. roqueforti	0	0	26.6	148.5	179.5	219.5
"Sterile mycelium"	0	0	147.5	193.6	253.5	260.2

The average dry-weight of the control mycelia grown in cherry juice was 268.8 mg.

The effect of the culture filtrate of *Penicillium expansum* was tested also in higher dilutions. At a dilution of 1/128 still no growth was observed, at a dilution of 1/256 the dry-weight of the mycelium appeared to be 55.0 mg, and at a dilution of 1/512 it was 119.0 mg.

The culture filtrate of *Penicillium spiculisporum* caused in *Thielaviopsis* an entirely abnormal development; in most instances the mycelium formed a thin film at the bottom of the flask.

In the second set of experiments the antagonists were cultivated in a potato extract instead of in cherry juice. This medium is approximately neutral (pH 6.7–6.9) and rich in carbohydrate, whereas cherry juice is acid (pH $\pm$ 4.5) and poor in carbohydrate. The results of these experiments are given in Table 2.

TABLE 2

Dry-weight in mg of the mycelium of *Thielaviopsis basicola* developed in undiluted and diluted filtrates of the potato-extract medium in which one of the listed antagonists had been cultivated

Antagonists	Degree of dilution of the filtrate					
	1	1/2	1/4	1/8	1/16	1/32
A. fumigatus I	0	0	0	0	100.0	176.7
A. fumigatus II	0	0	0	94.0	172.1	184.5
P. spiculisporum	0	0	41.5	55.1	73.5	101.7
P. spinulosum	0	0	0	104.5	138.5	146.5
P. expansum	0	0	0	0	0	0
G. roseum	0	81.8	112.2	153.5	223.0	201.0
P. roqueforti	0	0	0	0	0	172.1
"Sterile mycelium"	0	55.7	132.5	181.0	215.0	224.5

The average dry-weight of the control mycelia grown in potato extract was 235.3 mg.

Here too the effect of the culture filtrate of *Penicillium expansum* was tested in higher dilutions, viz. in 1/64, 1/128, 1/256 and 1/512; the dry-weights proved to be 112.0 mg, 142.6 mg, 138.2 mg and 154.3 mg respectively. In comparing these results with those obtained with the culture on cherry juice it is clear that this antagonist produces a more active filtrate when it is grown in cherry juice than when it develops in potato extract; dilution of the filtrate of the cherry juice medium to 1/512 has about the same effect as dilution of the filtrate of the potato-extract medium to 1/64.

In the filtrates of the cultures of *Penicillium spiculisporum* in the potato-extract medium the development of *Thielaviopsis* was, just as in those of the cherry-juice cultures, of an abnormal type. The mycelium at the surface of the culture liquid was irregular, dark, slimy and watery, and showed clear indications of lysis. The control mycelia were at the surface of the medium dry and woolly, and showed a regular type of growth.

Noteworthy is the strong increase shown by the antagonistic activity of *Penicillium roqueforti*. With the other antagonists, with the exception of *Gliocladium roseum*, the production of antibiotics was in potato extract less than in cherry juice.

4.3. Production of antibiotics in a Czapek-Dox medium

In the previous experiments the antagonists were cultivated in crude plant extracts. It seemed worth while to investigate whether antibiotics would also be produced by these fungi when they were grown in a medium composed of chemically pure substances. So far investigators interested in the production of antibiotics have mostly made use of this kind of media, especially of the Czapek-Dox medium, to which occasionally stimulating substances were added (Birkinshaw and Raistrick, 1931; Wiesner, 1942; Waksman, Horning and Spencer, 1943; Katzman *et al.*, 1945; a.o.). For this reason it seemed appropriate to carry out a number of supplementary experiments with antagonists cultivated in this medium. The antagonists that were used for this series of experiments were *Penicillium roqueforti*, *Penicillium spiculisporum*, *Penicillium expansum*, *Aspergillus fumigatus* and *Penicillium spinulosum*. The method of filtering differed from that used in the preceding experiments in so far that instead of a Seitz filter a glas filter was used. An advantage of the latter is that it does not absorb antibiotics, which in the case of the Seitz filter is not entirely excluded (Van Luyk, 1938).

Each antagonist was inoculated in a series of 3 Erlenmeyer flasks with a capacity of 300 cc, each containing 50 cc of the Czapek-Dox medium. The inoculated flasks were kept for 17 days in an incubator at a temperature of 24° C, after which the culture liquid was filtered through filter-paper and subsequently sucked through a glas filter (17 G4). The cultures of *Penicillium spiculisporum* were kept in the incubator for 20 days, because this fungus grows rather slowly.

As preliminary experiments had shown that *Thielaviopsis basicola* does not grow in the Czapek-Dox medium, the culture filtrates of

the antagonists were mixed with cherry juice. This mixture was transferred by means of a pipette to sterilized test tubes which subsequently were inoculated with *Thielaviopsis*. For each dilution of the filtrate 5 tubes were used. Moreover, 3 series of controls were installed, viz. (1) with cherry juice, (2) with 3 parts of cherry juice to 1 part of Czapek-Dox, and (3) with 50 parts of cherry juice to 1 part of Czapek-Dox. The Czapek-Dox medium was added to the culture liquid of the controls because the culture liquid in the other tubes too consisted partly of this medium. The tubes were kept in the incubator for 8 days, after which the growth of the mycelium in the various tubes was compared; this was done at sight, and the results, expressed by means of the symbols 0, +, ++ and +++, are reproduced in Table 3. In the three series of control cultures the growth of *Thielaviopsis* was nearly the same.

TABLE 3

Growth of *Thielaviopsis basicola* in a diluted filtrate of the Czapek-Dox medium in which one of the listed antagonists had been cultivated, and to which subsequently cherry juice had been added

Antagonists	Degree of dilution of the filtrate							pH of undiluted filtrate
	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
P. roqueforti	+++	+++	+++	+++	+++	+++	+++	7.85
P. spiculisporum . . .	0	0	0	0	0	+	++	4.65
P. spinulosum	0	0	0	0	+	++	+++	5.90
P. expansum	0	0	0	0	0	++	+++	4.10
A. fumigatus	0	0	0	0	0	+	+	4.70

pH of the Czapek-Dox medium 6.15.

Explanation of symbols: 0 = no growth; + = very slight growth; ++ = growth about half as strong as that of the controls; +++ = approximately the same growth as in the controls.

As appears from Table 3, no or almost no antibiotics were formed in the Czapek-Dox medium by *Penicillium roqueforti*. With a dilution of the filtrate to 1/2 or 1/4 the growth of *Thielaviopsis* was even better than in the control cultures, but when the filtrate was applied in higher dilutions, the growth of the fungus decreased. It is, therefore, not impossible that *Penicillium roqueforti* produces substances which exercise a stimulating effect on the growth of *Thielaviopsis*.

In Table 4 the antagonistic activity of filtrates obtained from cultures of four of the antagonists in the Czapek-Dox medium is compared with that of filtrates obtained from cultures of the same antagonists in cherry juice and in potato extract. In this table, instead of expressing the dilution by means of fractions, use has been made of "dilution figures", e.g. instead of $^1/_{64}$ the figure 64. The "dilution figures" indicate the proportion between the volume of the diluted solution and the undiluted one from which it was prepared.

Table 4 shows that the antagonistic activity of *Penicillium spiculisporum* is approximately 16 times stronger when this fungus is grown in the Czapek-Dox medium than when it develops in cherry juice,

and approximately 32 times stronger than when it is cultivated in potato extract. The decrease in the amount of antibiotics produced in the two last-mentioned media, may be due perhaps to the presence in these crude plant extracts of substances which influence the metabolism of this fungus in such a way that a smaller amount of antibiotics is produced.

TABLE 4

Growth inhibition caused in *Thielaviopsis basicola* by filtrates of three different media in which four different antagonists had been cultivated. The inhibiting effect is expressed by means of two figures, the first indicating the dilution at which the growth of the fungus is still entirely suppressed, the second that at which growth just becomes possible

	A. fumigatus	*P. spinulosum*	*P. expansum*	*P. spiculisporum*
Czapek–Dox	64–128	32–64	64–128	64–128
Cherry juice	16– 32	4– 8	128–256	4– 8
Potato extract	8– 16	4– 8	32– 64	2– 4

The data of this table have been taken from the tables 1–3.

The antibiotic activity of *Aspergillus fumigatus* and of *Penicillium spinulosum* too appeared to be stronger when these fungi were grown in the Czapek-Dox medium. *Penicillium expansum*, on the other hand, developed its highest antibiotic activity when it was grown in cherry juice; in the Czapek-Dox medium its antibiotic activity was lower, and in the potato extract it was once more somewhat lower. Among the fungi that are not mentioned in Table 4, *Penicillium roqueforti* did not produce antibiotics when grown in the Czapek-Dox medium or in cherry juice, but it developed a strong antagonistic activity in the potato extract.

It appears therefore that the antagonistic activity of each of the fungi mentioned above has its own character, and that the demands they make on the medium in order to reach their highest output of antagonistic substances, differ for each of them.

4.4. Influence of the carbon source in the culture medium on the production of antibiotics

Fungi are heterotrophic organisms, which means that they are with regard to their carbon requirements dependent upon the organic compounds that are present in their environment. The nature of the carbon source and the amount in which it is available, will therefore play an important part in their metabolism, and indirectly these factors will exercise their influence on the activities of the fungi. For the production of antibiotics the importance of the carbon source has already been pointed out e.g. by Van Luyk (1938), Lochhead, Chase and Landerkin (1946) and Florey *et al.* (1949), and is now generally recognized. However, it seemed worth while to study this problem by using *Penicillium roqueforti* and *Penicillium spiculisporum*, because these two fungi were so far not known as anta-

gonists. This study concerns the influence exercised (a) by the nature of the carbon source, and (b) by its concentration.

4.4.1. *Nature of the carbon source*

In these experiments use is made of a synthetic culture solution which as to its inorganic constituents agreed with a Czapek medium. This inorganic part of the culture liquid was pipetted into a number of Erlenmeyer flasks with a capacity of 500 cc; each of these flasks subsequently received a different carbohydrate as carbon source. These carbohydrates were glucose, maltose, saccharose, galactose, lactose, soluble starch, dextrin, fructose, maltose + casein, and brown sugar. In this way ten different culture media were obtained which differed only in the nature of the carbon source. In each of these media the carbon source constituted 3 % of the total; in the case in which the carbon source consisted of maltose mixed with casein 2 % was formed by the maltose and 1 % by the casein.

For each medium 4 Erlenmeyer flasks were used, and each flask received 50 cc of the medium. After being sterilized they were inoculated with one of the fungi.

The first set of experiments was carried out with *Penicillium roqueforti*. The flasks that had been inoculated with this fungus were kept for 17 days in an incubator at a temperature of 24° C.

Although there appeared to be much difference in the way in which *Penicillium roqueforti* developed in the various media, there was only one medium, viz. that containing lactose, which proved to be unsuitable for the cultivation of this fungus. At the end of the incubation period only a very slight growth was found in this medium, and for this reason these cultures had to be discarded. In the medium containing saccharose it showed a luxuriant but irregular growth, the surface of the mycelium being wavy, blotched with green patches and covered with droplets of a yellowish-brown liquid. In the media containing soluble starch the mycelium needed nearly a week to develop the green colour which in the media containing other carbohydrates developed almost immediately; however, at the end of this period the surface proved to be uniformly green; the exudation of droplets was here very strong. This exudation occurred also in the other media. The colour which the culture liquid finally assumed, differed considerably, viz. from light yellow to dark red.

At the end of the period of 17 days during which the cultures were kept in the incubator, the culture liquid of the various flasks was filtered in the usual manner, and just as in the preceding experiments various dilutions were prepared by mixing the filtrate with sterilized cherry juice. These dilutions were transferred to sterilized test tubes, 5 tubes being used for each dilution, and just as in the preceding series of experiments three sets of controls were instituted; in one set the medium consisted of cherry juice alone, in the two other sets of cherry juice mixed with a different proportion of the synthetic medium. The tubes were inoculated in the usual way with *Thielaviopsis*. After 8 days the growth of the latter in the various sets

was compared. The results are given in Table 5, in which the same symbols have been used as in Table 3. Here too in the three sets of controls the growth appeared to be nearly the same.

TABLE 5

Growth of *Thielaviopsis basicola* in culture filtrates obtained from cultures of *Penicillium roqueforti* in media differing in the nature of the carbon source; the filtrates were diluted with cherry juice

Carbon source	Degree of dilution of the filtrate						pH of medium	pH of undiluted filtrate
	1/2	1/4	1/8	1/16	1/32	1/64		
1. galactose . . .	0	+	+++	+++	+++	+++	6.00	7.90
2. fructose . . .	0	0	0	++	+++	+++	5.50	6.60
3. glucose . . .	+++	+++	+++	+++	+++	+++	5.80	8.65
4. saccharose . .	0	0	0	0	0	0	6.10	6.05
5. brown sugar .	0	0	0	0	+	++	6.00	6.85
6. maltose . . .	0	0	0	+	++	+++	5.80	7.10
7. soluble starch .	+	+++	+++	+++	+++	+++	6.35	8.55
8. dextrin	+++	+++	+++	+++	+++	+++	6.10	8.70
9. maltose+casein	+++	+++	+++	+++	+++	+++	5.50	8.75

Explanation of symbols, see Table 3.

It appears from Table 5 that the nature of the carbon source exercises a marked influence on the production of antibiotics by *Penicillium roqueforti*. In the medium containing saccharose this fungus developed by far its highest antagonistic activity. Even in the highest dilution of the filtrate that was tested in this series of experiments, viz. 1/64, the growth of *Thielaviopsis* proved to be completely suppressed. Afterwards the experiment was repeated with a dilution of $^1/_{128}$, and then a very slight growth was observed.

In the media containing monosaccharides, viz. glucose, galactose and fructose, as well as in those containing the disaccharides saccharose, maltose and brown sugar the antagonistic activity proved to differ.

The fact that in the medium containing glucose no growth-inhibiting substances were produced, whereas in the one containing fructose a fair amount, and in that with saccharose by far the largest quantity of these substances was produced, is doubtless unexpected. With regard to saccharose two possibilities should be considered, viz. that this substance is assimilated directly or that it is first hydrolysed. As in the latter case a mixture of equal parts of glucose and fructose would result, the fact that the antibiotic activity in the medium containing saccharose surpasses that in the medium containing fructose, could be explained only by assuming that the presence of one of these substances stimulates the influence exercised by the other. That the presence of one sugar may influence the utilization of another one, was shown by Horr (1936). Lilly and Barnett (1953) too point out that "the behaviour of fungi in the presence of mixed sugars is not always predictable from their behaviour on the single sugars comprising the mixture." However, there

is still an entirely different explanation, viz. that the three sugars (glucose, fructose and saccharose) undergo changes during the autoclaving (BARNETT, LILLY and WATERS, 1953). Lilly and Barnett state that "fructose darkened more than glucose when autoclaved. It is presumed that fructose is altered more by this method of sterilization than are the aldo sugars". Preliminary experiments of these investigators indicated that an inhibitory substance(s) is formed when fructose is autoclaved with the other constituents of the medium.

Another remarkable fact is that in the medium containing a mixture of maltose and casein as carbon source, no antiobiotics were produced, whereas in a medium in which the carbon source consisted of maltose alone, there were clear indications that a production of such substances actually took place. It seems plausible to assume that *Penicillium roqueforti* is influenced by the presence of casein in such a way that it produces little or no antiobiotics. That in the medium which contained brown sugar, a smaller amount of growth-inhibiting substance was produced than in one containing pure saccharose, may be due to the presence in the brown sugar of substances with a similar effect.

A similar set of experiments was performed with *Penicillium spiculisporum*. This fungus was cultivated in Czapek media with different carbon sources, the incubation period being 20 days. The substances used as carbon source were the same as in the experiments with *Penicillium roqueforti*, and they were used in the same concentration. The best growth was observed in the media containing glucose, maltose, saccharose, galactose and brown sugar. In the medium containing lactose growth was so insignificant that it did not seem worth while to prepare a filtrate. In the remaining media the development was fair, although much slower than in the first-mentioned ones.

The fact that *Penicillium roqueforti* as well as *Penicillium spiculisporum* show with lactose as carbon source but a very weak growth, whereas they grow well with glucose or with galactose, i.e. with the two monosaccharides that are formed when lactose is hydrolysed, is doubtless due to the circumstance that these two fungi are unable to hydrolyse this disaccharide to an appreciable extent. LILLY and BARNETT (1953) also arrived at the conclusion that "lactose is a poor source of carbon for fungi." They cultivated a number of fungi, one of which was *Penicillium spiculisporum*, on a medium containing lactose as carbon source as well as on one containing the two sugars that are formed when lactose is hydrolysed, and concluded from their experiments that "fungi failing to grow on lactose are unable to hydrolyse this sugar."

The culture filtrates of *Penicillium spiculisporum* were almost colourless, except with the medium containing brown sugar and in that with fructose, where the colour was light yellow. In order to test their antibiotic activity on *Thielaviopsis* they were diluted with sterilized cherry juice. The results of the experiments are summarized in Table 6.

In these experiments the culture filtrates of *Penicillium spiculisporum* developed a much stronger antibiotic activity than those of *Penicillium roqueforti* did in the preceding set, stronger, moreover, than would have been expected. In two instances, viz. in the filtrates of the media containing glucose and maltose, even the highest dilution that was

TABLE 6

Growth of *Thielviopsis basicola* in filtrates obtained from cultures of *Penicillium spiculisporum* in Czapek media differing in the nature of the carbon source; the filtrates were diluted with cherry juice

Carbon source	Degree of dilution of the filtrate						pH of undiluted filtrate
	1/8	1/16	1/32	1/64	1/128	1/256	
1. galactose	0	0	0	+	++	+++	6.10
2. glucose	0	0	0	0	0	0	4.60
3. fructose	0	0	0	+	++	+++	5.05
4. saccharose	0	0	0	0	+	++	4.70
5. brown sugar	0	0	0	0	0	+	4.75
6. maltose	0	0	0	0	0	0	4.60
7. soluble starch	0	0	0	0	+	++	5.80
8. dextrin	0	0	0	0	0	+	4.70

Explanation of symbols, see Table 3.

tested, viz. 1/256, caused a complete inhibition of the growth of *Thielaviopsis*. Afterwards the filtrates of these cultures were tested also in higher dilutions. In the dilution 1/512, the filtrate from the maltose culture proved to allow about half the normal growth, and in the dilution 1/1024 a normal one, whereas by the filtrate from the glucose culture in the dilution 1/512 still all growth was inhibited, and in the dilution 1/1024 half the normal growth was allowed.

Although the amounts of antibiotics that were produced by *Penicillium spiculisporum* in media with different carbon source, proved to differ, in all the media that were used in this set of experiments, such substances nevertheless were formed, and in this respect the results of these experiments differ therefore from those that were obtained in the experiments with *Penicillium roqueforti* (Table 5), in which no antibiotics were found in the filtrates of cultures containing glucose, dextrin or the mixture of maltose and casein. Other noteworthy differences are that *Penicillium spiculisporum* develops in the medium containing brown sugar a stronger antibiotic activity than it does in the medium containing pure saccharose, and that the proportion between the amounts produced in the medium containing saccharose and in the media which contained the substances that arise when the latter is hydrolysed, was as might be expected when we assume (1) that the saccharose is hydrolysed before it is assimilated and (2) that the products of the hydrolyzation do not influence the utilization of each other.

4.4.2. *Concentration of the carbon source*

The experiments of which the results were summarized in Table 5, have shown that in the case of *Penicillium roqueforti* saccharose is the

most suitable carbon source for the production of antibiotics. In these experiments the culture medium contained 3 % saccharose, but as it seemed possible that a change in the concentration of the saccharose might give different results, a number of experiments have been performed in which this concentration was varied.

In the same way as in the earlier experiments with *Penicillium roqueforti* here too a medium was used which contained the same inorganic constituents as the Czapek one. With this medium 5 Erlenmeyer flasks were filled, each flask receiving 200 cc and in addition a definite weight of saccharose; in this way saccharose concentrations were obtained of 1 %, 2 %, 3 %, 4 % and 5 %. The pH of the media proved to vary between 6.05 and 6.10. Each of these liquids was distributed over 4 Erlenmeyer flasks, so that each of the latter received 50 cc. After sterilization the flasks were inoculated with *Penicillium roqueforti*.

The growth of the fungus differed but slightly in the various media; in that with 1 % saccharose, however, it remained behind. The exudation of droplets was strong in the media with 1 %, 2 % and 3 % saccharose, but less strong in those with 4 % and 5 %; in the medium with 1 % saccharose they were colourless, and in those with 2 % and 3 % of a characteristic chestnut-brown.

After an incubation period of 17 days the culture medium was filtered. The filtrate of the medium with 1 % saccharose proved to be yellow-brown, that of the other ones cherry-red. After dilution with cherry juice the antibiotic activity of the filtrate was tested in the usual way on *Thielaviopsis* (Table 7).

TABLE 7

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of *Penicillium roqueforti* in Czapek media containing from 1 % to 5 % saccharose; the filtrates were diluted with cherry juice

Concentration of saccharose	Degree of dilution of the filtrate					pH of undiluted fiiltrate
	1/16	1/32	1/64	1/128	1/256	
1 %	+++	+++	+++	+++	+++	8.10
2 %	0	++	+++	+++	+++	7.95
3 %	0	0	0	++	+++	6.45
4 %	0	0	0	+	++	5.85
5 %	0	0	0	0	++	5.90

Explanation of symbols, see table 3.

In these experiments the antagonistic activity of the filtrate appeared to increase regularly with the increase of the sugar concentration in the culture medium. The experiments confirmed the conclusion reached in the earlier ones according to which saccharose is to be regarded as a carbon source which allows this fungus to develop a strong antagonistic activity; they also confirmed the impression created by the earlier experiments that the development of a strong antagonistic activity is accompanied by a comparatively low pH.

The results obtained by cultivating the antagonist in media con-

taining saccharose, however, are not always the same. In the next section some experiments will be described which prove that, judged by the antagonistic activity of the culture filtrate, the utilization of the saccharose may be severely hampered by the presence of other organic compounds in the medium.

4.5. Comparison of the antagonistic activity of filtrates obtained from cultures of Penicillium roqueforti in potato extract and in a Czapek medium

The experiments with *Penicillium roqueforti* in media which differed in the nature of the carbon source, have shown that this fungus does not produce antibiotics in media in which glucose is the only carbon source, whereas in media containing saccharose as the only carbon source, the production of antibiotics is highest (Table 5). Earlier in this work it appeared, however, that a high antagonistic activity was also developed when the fungus was cultivated in potato extract to which 2 % glucose had been added (Table 2). In a medium with soluble starch as the only source of carbon, on the other hand, hardly any antagonistic activity was developed, so that the strong antagonistic activity reached in the potato extract can not be due, at least not directly, to the starch that is present in the latter. It looks therefore as if the potato extract contains one or more other substances which affect the production of antibiotics. In order to study the effect of such substances on the utilization of glucose and saccharose a set of experiments were carried out in which *Penicillium roqueforti* was cultivated in potato extract and in a Czapek medium to which glucose or saccharose had been added. In these experiments 5 media were used of which the composition is given in Table 8. With each medium three cultures were made, and at the end of 17 days all the cultures were filtered, and the filtrates diluted with various amounts of potato extract. The antagonistic activity of these dilutions was tested in the usual way on *Thielaviopsis*. The results are given in table 8.

It appears that the antagonistic activity decreased in a Czapek

TABLE 8

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of *Penicillium roqueforti* in various media; the filtrates were diluted with potato extract

Culture medium of *Penicillium roqueforti*	Degree of dilution of the filtrate					pH of undiluted filtrate
	1/8	1/16	1/32	1/64	1/128	
1. Czapek medium + 3 % glucose	+++	+++	+++	+++	+++	8.15
2. Potato extract + 3 % glucose	0	0	+	++	+++	5.90
3. Czapek medium + 3 % saccharose	0	0	0	0	++	5.93
4. Potato extract + 3 % saccharose. . . .	+++	+++	+++	+++	+++	6.50
5. A mixture of 3 and 4 . . .	+++	+++	+++	+++	+++	7.25

Explanation of symbols, see table 3.

medium with glucose and in a potato extract to which saccharose had been added, and that it increased in a Czapek medium with saccharose and in a potato extract with glucose. The filtrate of a mixture of the two media containing saccharose had no antagonistic activity.

Lilly and Barnett (1953) arrived at the conclusion that "the utilization of sugars (either alone or in mixtures) is modified by a number of factors, which include other constituents of the medium, the environment and time."

4.6. Influence of stimulants on the production of antibiotics

That some substances may exercise a stimulating influence on the activities of micro-organisms has already been known for a considerable time, and that the production of antiobiotics too may be affected by such substances has been shown by several investigators. Katzman *et al.* (1944) found that the production of antibiotics by *Aspergillus clavatus* is favoured by "corn steep liquor"; they recommend to add 2 cc of this substance to one liter of the culture medium. Lochhead, Chase and Landerkin (1946), on the other hand, found that the production of antibiotics by two different species of *Penicillium* was depressed by the presence of corn steep liquor. Moyer and Coghill (1946a), however, report that the production of penicillin in a Czapek-Dox medium is increased 6 to 8 times when so much corn steep liquor is added that the medium consists for 7.5 % to 10 % of this extract.

In the next set of experiments the influence of commercial corn steep liquor was studied once more. The experiments were made with *Penicillium roqueforti*, and different concentrations of the corn steep liquor were tried.

Five Erlenmeyer flasks received each 200 cc of a Czapek medium with 5 % saccharose. To four of these flasks amounts of 2, 4, 8 and 12 cc corn steep liquor were added; the culture liquid in these flasks contained therefore respectively 1 %, 2 %, 4 % and 6 % of this extract; the fifth flask contained no corn steep liquor and served as a control. The contents of each flask were divided over 4 other flasks, and after sterilization the latter were inoculated with *Penicillium roqueforti*.

The fungus developed more luxuriously and far more rapidly in the media which contained the corn steep liquor than in the control flasks. When in the latter the first signs of growth became discernible, there was already a closed layer of mycelium at the surface of the medium in the other flasks. In the beginning this fleece had a light green colour, but in the media containing the higher concentrations of the corn steep liquor it afterwards became brown. When the cultures were 15 days old, there appeared to be a distinct gradation from the green colour in the cultures containing 1 % corn steep liquor to the brown one of the cultures which contained 6 % of this admixture. In the media containing this stimulating agent the myce-

lium is evenly coloured, whereas it shows patches of white in the controls. In the media containing corn steep liquor it forms, moreover, a thick and solid, strongly wrinkled fleece; and the thickness and solidity of the latter increases with the concentration of the corn steep liquor. Furthermore, the exudation of droplets is more abundant in the media with the corn steep liquor than in the controls.

At the end of a period of 15 days the culture liquid was filtered, and the filtrate diluted in the usual way with cherry juice. The antibiotic activity of these dilutions was tested by the aid of *Thielaviopsis* (Table 9).

TABLE 9

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of *Penicillium roqueforti* in a Czapek medium containing varying concentrations of corn steep liquor; the filtrates were diluted with cherry juice

Percentage of corn steep liquor	Degree of dilution of the filtrate					pH of medium	pH of undiluted filtrate
	1/32	1/64	1/128	1/256	1/512		
0	0	0	+	++	+++	6.30	6.75
1	0	+	++	++	+++	4.90	6.77
2	+++	+++	+++	+++	+++	4.70	7.76
4	+++	+++	+++	+++	+++	4.60	8.60
6	+++	+++	+++	+++	+++	4.50	8.60

Explanation of symbols, see Table 3.

Table 9 shows that the production of antibiotics underwent, in conformity with the findings of LOCKHEAD, CHASE and LANDERKIN (1946), a marked decrease in the media containing an admixture of corn steep liquor. Unfortunately we did not test the effect of concentrations higher than that of the dilution 1/32, but it seems nevertheless justified to assume that the growth-inhibiting effect of the filtrates decreases with an increase of the concentration of the corn steep liquor in the culture medium.

4.7. INFLUENCE OF THE INCUBATION TIME ON THE PRODUCTION OF ANTIBIOTICS BY PENICILLIUM ROQUEFORTI

In the experiments that so far have been described, the incubation period of *Penicillium roqueforti* always lasted from 15 to 20 days. However, it is not unthinkable that the amount of antibiotics which is produced in a given period, may show some relation to the rate of growth, and as the latter decreases after some time, the values found in these experiments do not necessarily give an entirely satisfactory picture of the production process. To test this possibility some experiments were made in which the incubation time was varied.

These experiments were performed with 18 Erlenmeyer flasks of 300 cc capacity, each containing 50 cc of a Czapek medium with 5 % saccharose as carbon source. This group of 6 times 3 flasks were inoculated with *Penicillium roqueforti*, and placed in an incubator at a temperature of 24° C. After 10 days the first group of 3 flasks were taken out of the incubator, and then with intervals of 5 days the other

groups were removed, the contents filtered, and the filtrates tested in the usual way by estimating their effect on the growth of *Thielaviopsis* (Table 10).

It seems that *Penicillium roqueforti* completes its growth under the circumstances of the experiment in about 10 days; in the subsequent period little or no further expansion was observed. At the end of these 10 days the culture filtrates appeared to have assumed already the cherry-red colour which is characteristic for this strain of the fungus, and the exudation of droplets had just started.

TABLE 10

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of *Penicillium roqueforti* of different age; the cultures were made in a Czapek medium containing 5 % saccharose, and the filtrates were diluted with cherry juice

Incubation time in days	Degree of dilution of the filtrate						pH of undiluted filtrate
	1/16	1/32	1/64	1/128	1/256	1/512	
10	0	0	0	+	++	+++	7.15
15	0	0	0	0	+	+++	5.90
20	0	0	0	0	+	+++	5.50
25	0	0	0	0	+	+++	5.40
30	0	0	0	0	+	+++	5.20
35	0	0	0	+	++	++	5.20

pH of the Czapek medium 6.20.
Explanation of symbols, see Table 3.

It appears from table 10 that *Penicillium roqueforti* needs about 15 days to produce the maximum amount of antibiotics; when the cultivation is continued for a longer period, no more antibiotics or at least no appreciable amounts of antibiotics are produced. It seems therefore that this fungus produces its antibiotics during its period of active growth. When the cultures were left in the incubator for 35 days, the amount of antibiotics in the culture liquid seemed to decrease. This decrease can not be due to instability of the antibiotics, as the latter appear to be comparatively stable (see Table 11). It seems more probable that the antibiotic activity decreases because of the presence of other metabolites in the culture medium or else because part of the antibiotics is absorbed by the fungus itself.

From various experiments the impression was gained that the production of antibiotics by the strain of *Penicillium roqueforti* that was used here, is remarkably constant, at least so long as the external conditions remain the same. With other antagonists this is not always so. With *Penicillium expansum*, for instance, Van Luyk (1938) noted a considerable degree of variability in the production of antibiotics.

4.8. Stability of the antibiotics in the culture filtrates

Several of the antibiotics are apparently not very stable, for the filtrates in which they are present, tend to lose their antagonistic activity to some extent, for instance when they are exposed to higher temperatures or simply when they are stored for some time (Weind-

LING and EMERSON, 1936; VAN LUYK, 1938; WEINDLING, 1941; BRIAN and McGOWAN, 1945; BRIAN, CURTIS, HEMMING and McGOWAN, 1946; and others).

It seemed worth while to find out whether the antibiotics in the culture filtrates of *Penicillium expansum*, *Penicillium spinulosum*, *Aspergillus fumigatus*, *Penicillium spiculisporum*, *Penicillium roqueforti* and *Gliocladium roseum* are able to withstand a 10-minutes heat-sterilization at 103° C.

The antagonists were cultivated for 16 days in a Czapek-Dox medium at a temperature of 24° C, but as *Penicillium roqueforti* does not produce antibiotics in this medium, for this species a Czapek medium was used to which 5 % saccharose had been added, and as *Penicillium spiculisporum* is a slow grower, the latter was kept three days longer in the incubator. At the end of the incubation period the culture media were filtered through filter-paper, and the filtrates divided in two parts; one part was placed in an autoclave and exposed for 10 minutes to a temperature of 103° C, whereas the other part was sucked through a glass filter. Of both parts dilutions were made with cherry juice, and the antagonistic activity of the filtrates were tested in the usual way on *Thielaviopsis*. Cultures of this fungus on cherry juice without an admixture of filtrate were used as controls.

It appeared that the filtrates of *Aspergillus fumigatus* and of *Gliocladium roseum* had lost in the autoclave about half their antagonistic activity, whereas the antibiotic activity of the other filtrates showed no decrease.

The possibility that the antibiotic activity of the culture filtrates might decrease in the course of time, has also been investigated. In these experiments the same antagonists were used as in the preceding ones with the exception of *Gliocladium roseum*, and here too they were cultivated in a Czapek-Dox medium to which in the case of *Penicillium roqueforti* 5 % saccharose had been added. The incubation time and the incubation temperature too were the same.

The culture filtrates and the dilutions of the latter with cherry juice were prepared in the usual way, and each dilution was divided over four parallel series. One of the latter was inoculated immediately with *Thielaviopsis*, wheares the three other ones were stored, together with controls, at room temperature. The second, third and fourth series were inoculated respectively on the 16th, the 31st and the 46th day. The results of the tests are recorded in Table 11.

On the whole the culture filtrates retained their antibiotic activity in the period of 45 days during which they were stored. In that of *Aspergillus fumigatus*, however, it decreased in 30 days to about half its original value.

In the filtrate of *Penicillium roqueforti* that had been diluted to 1/128, after 15 days storage only a slight growth of *Thielaviopsis* was noticeable, and in the filtrate that had been diluted to 1/256, after the same period of storage growth reached about half the normal rate. It looks, therefore, as if the antibiotic activity of the filtrate had increased. In the filtrates that had been stored for 30 and 45 days, the growth of *Thielaviopsis* seemed to have decreased even somewhat

further. It is not impossible that this increase of the antibiotic activity of the filtrate may be due to a loss of water by evaporation. i.e. to an increase in the concentration of the solution. That it would be accidental, i.e. a result of the variability of *Thielaviopsis*, is hardly believable.

TABLE 11

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of various antagonists and stored for a different length of time; the filtrates were diluted with cherry juice

Name of antagonist and pH of filtrate	Storage in days	Degree of dilution of the filtrate				
		1/32	1/64	1/128	1/256	1/512
Penicillium expansum 4.10	0	0	0	++	+++	+++
	15	0	+	++	+++	+++
	30	0	+	++	+++	+++
	45	0	+	++	+++	+++
Penicillium spinulosum 5.90	0	0	+	++	+++	+++
	15	0	+	++	+++	+++
	30	0	+	++	+++	+++
	45	0	+	++	+++	+++
Aspergillus fumigatus 4.70	0	0	0	+	+	++
	15	0	0	+	+	++
	30	0	+	+	++	+++
	45	0	+	+	++	+++
Penicillium spiculisporum 4.65	0	0	0	+	++	+++
	15	0	0	+	++	+++
	30	0	0	+	++	+++
	45	0	0	+	++	+++
Penicillium roqueforti 5.75	0	0	0	++	+++	+++
	15	0	0	+	++	+++
	30	0	0	+	++	+++
	45	0	0	+	++	+++

Explanation of symbols, see Table 3.

The loss of water by evaporation was estimated by comparing in the test tubes which contained the diluted filtrates, the average height of the liquid column at the beginning of the experiment and 30 days later. It appeared that the loss amounted to approximately 1/8 of the original volume. This loss might have been compensated by the addition of a corresponding volume of water, but this was not done because in that case the results obtained with this series would not have been fully comparable to those obtained with the other ones. At any rate, if this loss would explain the increase of the antibiotic activity, this increase would be spurious, and then the antibiotic activity of the culture filtrate of this fungus would have to be regarded as remarkably constant.

4.9. Influence exercised on the growth of Thielaviopsis basicola by culture filtrates mixed with an agar medium

So far the effect exercised by the culture filtrates of the antagonists

on *Thielaviopsis* was tested only in liquid media. A disadvantage of this method is that the effect can be determined only once, viz. at the end of the experiment, and not in the course of the latter. If the filtrates are dissolved in a molten agar medium, the growth of the *Thielaviopsis* colony which develops on the congealed agar, can be measured at various intervals, and in this way eventually the presence of changes in the antibiotic activity of the filtrates may be detected. It might be objected that the antibiotics are perhaps partly absorbed by the agar, a possibility that was already suggested by VAN LUYK (1938). SLAGG and FELLOWS (1947), however, cultivated a large number of soil fungi on media of this kind in order to estimate the antibiotic activity of the latter, and found the method most convenient.

The following set of experiments were carried out in order to study the behaviour of *Thielaviopsis* on agar media containing antibiotics. In order to obtain such a medium the antagonists were cultivated in cherry juice at a temperature of 24° C, and after 16 days the culture liquid was filtered in the usual way. Tubes containing 15 cc cherry-agar were sterilized and placed in a water bath at a temperature of 45° C, and then to each of the tubes 5 cc undiluted or diluted filtrate was added; in this way a series of dilutions ranging from 1/4 to 1/128 were obtained. The culture filtrate of *Aspergillus fumigatus* was further diluted to 1/256, and that of *Penicillium expansum* to 1/512. The contents of the tubes were transferred to Petri dishes and inoculated with *Thielaviopsis*. The inoculation was carried out by means of round discs of mycelium with a diameter of 3 mm. At the same time a series of controls was instituted which consisted of 15 cultures; the medium of the latter was prepared by adding instead of filtrate 5 cc sterilized cherry juice to the molten agar. The cultures were placed in an incubator and kept at a temperature of 24° C, and from time to time the linear growth of each colony was estimated. To this end the diameter of the colony was measured in two directions, one perpendicular to the other. From the diameter 3 mm, i.e. the diameter of the disc that was used for the inoculation, was subtracted.

The effect of each dilution was tested in five cultures, and in Table 12 the average diameter (minus 3 mm) reached by these colonies in the first five days and their subsequent increase in three periods of three days are recorded.

Table 12 shows that the increase in diameter of the *Thielaviopsis* colonies in the three consecutive periods of three days was approximately the same. It can certainly not be denied that the rate of growth fluctuated somewhat, e.g. in the tests with the culture filtrate of *Aspergillus fumigatus*, but there is no regularity in these fluctuations, and they may therefore probably be ascribed to accidental circumstances. In the tests with the culture filtrate of *Penicillium spinulosum*, however, the rate of growth appeared to increase fairly regularly. This phenomenon remains for the moment inexplicable; it might mean that *Thielaviopsis* is able to adapt itself to some extent to the presence of this filtrate.

On the whole *Thielaviopsis* showed itself very sensitive with regard

TABLE 12

Increase in diameter in mm shown by colonies of *Thielaviopsis basicola* cultivated on cherry-agar to which culture filtrates of antagonists had been added in various dilutions

Name of antagonist and pH of filtrate	Dilution of filtrate	Diameter reached in 5 days	Increase in diameter in 3 periods of 3 days			Average increase per period	Same as % of normal increase
			1	2	3		
Penicillium spiculisporum 5.20	1/4	0	0	0	0	0	0
	1/8	5.0	3.5	3.2	2.8	3.2	27
	1/16	8.5	2.5	2.5	2.5	2.5	21
	1/32	11 0	7 5	7.5	7.2	7.4	63
	1/64	12.0	8.0	7.5	8.0	7.8	66
	1/128	13.0	9.0	8.0	8.5	8.5	72
	Controls	16.0	12.0	11.7	11.5	11.7	100
Penicillium spinulosum 4.80	1/4	0	0	0	0	0	0
	1/8	0	0	0	0	0	0
	1/16	0	2.0	2.8	5.4	3.4	30
	1/32	1.5	2.0	2.5	5.0	3.2	28
	1/64	4.2	6.3	7.2	7.0	6.8	60
	1/128	9.4	7.8	8.3	9.2	8.4	74
	Controls	16.0	11.0	12.0	11.0	11.3	100
Aspergillus fumigatus 5.30	1/16	0	0	0	0	0	0
	1/32	0	1.7	1.5	0.8	1.3	12
	1/64	1.0	2.0	1.5	1.9	1.8	16
	1/128	3.3	4.7	4.0	5.2	4.6	41
	1/256	5.6	6.6	6.8	8.5	7.3	65
	Controls	16.0	11.0	12.0	11.2	11.4	100
Penicillium expansum 4.20	1/16	0	0	0	0	0	0
	1/32	0	0	0	0	0	0
	1/64	0	0	2.0	2.5	2.3	20
	1/128	4.0	7.0	7.0	7.4	7.1	63
	1/256	8.0	8.5	8.5	9.0	8.7	77
	1/512	12.0	9.5	9.7	10.1	9.8	87
	Controls	16.0	11.0	12.0	11.0	11.3	100
Penicillium roqueforti 5.50	1/4	6.5	7.0	8.0	8.5	7.8	69
	1/8	10.0	9.2	9.8	10.0	9.7	86
	1/16	12.5	10.5	11.0	11.0	10.8	96
	1/32	14.0	11.6	10.4	11.5	11.2	99
	1/64	15.0	11.6	11.6	11.8	11.7	103½
	1/128	15.0	11.5	11.7	11.8	11.7	103½
	Controls	16.0	11.0	12.0	11.0	11.3	100

to the antibiotics that were tested in these experiments. With an increasing dilution of the filtrate, the rate of growth of the test fungus gradually increased. The conclusion therefore seems justified that a solid culture medium may be regarded as a very suitable substrate for testing the antibiotic activity of culture filtrates.

It can, on the other hand, not be denied that *Thielaviopsis* sometimes showed deviations from its normal development. On media to which the to 1/8 or to 1/16 diluted culture filtrate of *Penicillium spiculisporum* had been added, the mycelium showed an abnormal aspect, and even

lysis of the hyphae, and the culture filtrates of *Penicillium spinulosum* and of *Aspergillus fumigatus* caused the development of so many white sectors in the colonies that the latter looked decidedly blotched. However, as the white sectors appeared to expand in the same way as the green ones, their presence will not have affected the results of the growth measurements.

When the figures for the increase in the test cultures expressed as a percentage of the increase in the control cultures are compared with each other, it appears that the culture filtrate of *Penicillium expansum* had the strongest antibiotic effect. Even in a dilution of 1/32 it completely inhibited the growth of *Thielaviopsis*. With the culture filtrates of *Aspergillus fumigatus*, *Penicillium spinulosum* and *Penicillium spiculisporum* a total growth inhibition required a concentration corresponding to a dilution respectively to 1/16, 1/8 and 1/4. With the culture filtrates of *Penicillium expansum* at a dilution to 1/128 a growth corresponding to 63 % of that found in the control cultures was reached, whereas with the culture filtrates of *Aspergillus fumigatus* a similar growth required a dilution to 1/256, with those of *Penicillium spinulosum* a dilution to 1/64, and with that of *Penicillium spiculisporum* a dilution to 1/32. The culture filtrate of *Penicillium roqueforti* exercised but a weak antibiotic action; with a dilution to 1/4 the growth of *Thielaviopsis* still amounted to 69 % of the value found in the control cultures, whereas at a dilution of 1/32 the difference between the test culture and the control had entirely disappeared.

4.10. Production of antibiotics by various strains of Penicillium roqueforti

Although the various strains belonging to the same fungus species show, as a rule, little or no difference in aspect, there is sometimes a considerable difference in behaviour. Such a difference is recognizable, for instance, in the tables 1 and 2 in the production of antibiotics by two strains of *Aspergillus fumigatus*, and similar examples have frequently been given in the literature (Van Luyk, 1938; Jaarsveld, 1942; a.o.).

In the following series of experiments the production of antibiotics by the strain of *Penicillium roqueforti* that so far had been used in the experiments, was compared with that of five strains belonging to the culture collection of the "Central Bureau of Fungus Cultures (C.B.S.)" at Baarn (Netherlands). These five strains had been incorporated in the collection in the years 1929, 1930 and 1938. A strain isolated from French roquefort cheese was tested too.

Of each strain three cultures were made in a Czapek medium containing 3 % saccharose, and at the end of an incubation period of 17 days the culture liquid was filtered, and the antibiotic activity of the filtrate in dilutions starting with 1/4 tested on *Thielaviopsis*.

The filtrate obtained from the strain that in the course of this investigation was isolated from soil, caused at a dilution of 1/64 a complete inhibition of the growth of *Thielaviopsis*, whereas at a

dilution of 1/128 a slight growth of the test fungus was noticeable. The filtrate of one of the strains received from the "C.B.S." allowed a slight growth at a dilution of 1/4 and normal growth at one of 1/8. The filtrates of the remaining strains caused no inhibition at all. In the filtrates of three of the latter the growth of the test fungus was better in the less diluted than in the more diluted filtrates and even better than in the control cultures.

These experiments on the production of antibiotics by various strains, therefore, have clearly shown how considerably the strains may differ in this respect. The first-investigated strain is a good producer of antibiotics, whereas the other ones are in this respect of little or no importance.

4.11. The way in which different strains of thielaviopsis basicola react on antibiotics

The preceding section dealt with the way in which different strains of the same fungus species may differ in their production of antibiotics. Here the way in which different strains of the same test fungus react on the presence of these antibiotics, will be considered. That these strains may differ in this respect, was known already. Stover (1950a) found that the two "cultural types" of *Thielaviopsis basicola* which were isolated by him from tobacco, differed in their reaction on the presence of a bacterial antagonist indicated as "A". The brown "cultural type" proved to be strongly inhibited, while the mycelium of the grey one became submerged but its growth was not greatly retarded.

In order to find out whether there are differences in the way in which various strains of *Thielaviopsis basicola* react on the culture filtrate of *Penicillium roqueforti*, six of them were tested, of which 4 were obtained from the "C.B.S.". In cultures on cherry-agar these strains appeared to show different properties.

(1) The strain that was isolated by Mooi–Bok in 1949 from *Lathyrus odoratus*, grows slowly and stops growing when but a part of the agar surface is covered. The cultures are green at the bottom and white with much aerial hyphae at the upper side. Spores are produced but scarcely and often not at all.

(2) The strain that was isolated by Tiddens in 1933 from Poinsettia (*Euphorbia pulcherrima*), grows luxuriously with much aerial mycelium. The cultures are green, dark green to nearly black at the bottom and greyish at the upper side. In comparison with the luxurious production of mycelium the production of spores is but scanty.

(3) This strain was received from America where it was isolated by Gilbert from tobacco; it is since 1926 in the collection of the "C.B.S.". The properties of the cultures agree with those observed in the strain that was isolated from Poinsettia. Its growth is luxurious with much aerial mycelium, but the colour of the cultures is darker, and more spores are produced. There is a tendency to form sectors.

(4) The cultures of this strain, which was isolated in 1948 by Van Holder from *Cypripedium* roots, remain appressed to the agar

substrate and develop less aerial mycelium. At the bottom the cultures are dark green and at the upper side light green. A large amount of endoconidia and of chlamydospores are produced.

(5) The strain that was isolated from the roots of *Primula obconica* grows slowly, and the mycelium remains appressed to the agar substrate and produces but few aerial hyphae. The cultures are dark green at the bottom and green at the upper side. Endocondidia and chlamydospores are produced in very large numbers. There is a tendency to produce sectors.

(6) The strain that was isolated from the roots of *Nicotiana glutinosa* grows rapidly and produces much aerial mycelium, endoconidia and chlamydospores. The cultures are dark green at the bottom and green at the upper side. Here too there is a tendency to sector forming.

The antagonist, *Penicillium roqueforti*, was cultivated in a Czapek medium containing 5 % saccharose, and of the culture filtrate 6 parallel series of dilutions with cherry juice were made, i.e. one series for each of the *Thielaviopsis* strains. The growth of the latter in the media consisting of cherry juice with various amounts of the culture filtrate of the antagonist was compared with its growth in control cultures on cherry juice without any addition of culture filtrate (Table 13).

TABLE 13

Growth of various strains of *Thielaviopsis basicola* in culture filtrates of *Penicillium roqueforti* that had been diluted with cherry juice

Strains of *Thielaviopsis basicola* isolated from:	Degree of dilution of the filtrate				
	1/32	1/64	1/128	1/256	1/512
Lathyrus odoratus	0	0	0	++	+++
Euphorbia pulcherrima	0	0	0	+++	+++
Nicotiana tabacum	0	0	0	+++	+++
Cypripedium spec.	0	0	+	++	+++
Primula obconica	0	0	0	++	+++
Nicotiana glutinosa	0	0	0	++	+++

Explanation of symbols, same as in Table 3.

The strain that had been obtained from the roots of *Cypripedium* showed some growth in the cultures which contained the filtrate in the dilution 1/128, but it was a very slight one. On the whole the various strains seem to be affected in the same way by the culture filtrate of *Penicillium roqueforti.*

So far the production of antibiotic substances by a number of soil fungi and the effect of these substances on the growth of *Thielaviopsis basicola* were studied in vitro. It appeared from the experiments that the growth-inhibiting activity of the filtrates obtained from the cultures of the various antagonists differed widely, and that the kind of culture medium and the nature and concentration of the carbon source exercised a considerable influence on the activity of the filtrates. The growth-inhibiting substance in the culture filtrates, with the exception of that in the filtrate of *Aspergillus fumigatus*, proved to be

resistent against steam sterilization for ten minutes at 103° C, and it was not affected by storage at room temperature for a period of 45 days.

Of the culture filtrates obtained from the antagonists that originally had been selected, those of *Gliocladium roseum* and of the sterile mycelium showed but a weak antibiotic activity, and these fungi were therefore discarded. The experiments with *Penicillium roqueforti*, on the other hand, looked promising. It appeared that the antibiotic activity of the culture filtrates of this fungus remained constant under constant environmental conditions. However, when this fungus was cultivated in media of different composition, the antibiotic activity proved to vary considerably. Because of this sensitivity to the composition of the medium *Penicillium roqueforti* was not used in the experiments on the behaviour of *Thielaviopsis* in soil which will be dealt with in the last chapter.

The antibiotic activity of the culture filtrates obtained from the other antagonists, viz. from *Aspergillus fumigatus*, *Penicillium spinulosum*, *Penicillium expansum* and *Penicillium spiculisporum*, showed less variability under differing circumstances. *Penicillium expansum* appeared to be the antagonist with the highest activity.

5. DEGREE OF SURVIVAL OF THELAVIOPSIS BASICOLA IN UNSTERILIZED SOIL

5.1. Relation between the natural microflora and the thielaviopsis population during a period of systematic infestation

It is well-known that many pathogenic fungi occurring in the soil, disappear in the long run or decrease in number (Katznelson, 1940). Physical factors and the activity of antagonistic micro-organisms are usually held responsible for this decrease. The fact that *Thielaviopsis basicola* is rather regularly found in soils (Yarwood, 1946; Stover, 1950b), proves that this root parasite does not disappear easily. It would therefore be interesting to know to what extent *Thielaviopsis* can maintain itself in a naturally infested soil as well as in a soil to which from time to time cultures of this fungus are added.

An investigation carried out to find an answer to this question would have to consider the following points:

(1) Does the number of *Thielaviopsis* colonies that can be isolated from a soil in which this fungus is naturally present, undergo a decrease in the course of time?

(2) What influence does a periodical addition of *Thielaviopsis* material exercise on the density of the population of the soil fungi that originally was present?

(3) What influence does such a periodical addition of *Thielaviopsis* exercise on the frequency of its antagonists?

(4) What is the influence which a periodical addition of *Thielaviopsis* to the soil exercises on the number of colonies of this fungus that can be isolated during this period of systematic infestation?

The two kinds of soil that were used in this series of experiments

were (a) a soil on which healthy plants of *Primula obconica* had been growing, and which consisted for 30 % of mud, for 20 % of leaf mould, for 20 % of clay, for 20 % of hog's dung and for 10 % of peat-dust; as no diseased plants had been observed on this soil, it will be designated here with the name "healthy soil"; and (b) "diseased soil", a soil on which severely infected plants of *Primula obconica* had been found, and which contained remains of *Primula* roots which on account of their infection by *Thielaviopsis basicola* had assumed a brown colour; this soil consisted for 30 % of mud, for 20 % of leaf mould and for 50 % of clay.

With each of the two kinds of soil 10 pots with a capacity of 1000 cc were filled to 1 cm below the brim, and in order to obtain a favourable environment for the development of fungi, the soil was moistened with sterilized water. In order to restrict the intervention of fungi from without, the pots were covered with plates of glass. Each of the two sets of pots was divided into two groups, indicated as "A" and "B"; those belonging to the groups "B" were subsequently infested with *Thielaviopsis*. All the pots were placed in a greenhouse where the temperature fluctuated between 20° C and 25° C. In order to keep the moisture content of the soil at a favourable level, the pots were embedded in peat-dust which was watered from time to time.

For the infestation of the pots belonging to the B groups with *Thielaviopsis*, use was made of suspensions of mycelium and spores. These suspensions were obtained from cultures in potato extract with 2 % glucose; when the latter were 14 days old, the mycelium was separated from the culture liquid and washed; after that 25 cc sterilized water was added. In this way a dense suspension of mycelium and spores was obtained.

Each of the pots belonging to the B groups received every other day 25 cc of this suspension, and each pot belonging to the A groups received at the same time an equal volume of sterilized water. In this way the soil in the pots belonging to the B group was regularly enriched with *Thielaviopsis*, whereas nothing was done which could change the composition of the microbiological population in the pots belonging to the A groups.

The composition of the microbiological population in the soil of the various pots was studied by means of soil samples. The latter were taken with intervals of two days from the 2nd March to the 22nd March, and in the pots belonging to the B groups this was done before a new *Thielaviopsis* suspension was added. Six days after the last administration of a *Thielaviopsis* suspension, i.e. on the 28th March, the soils in the pots were sampled once more. The samples were taken about 2–3 cm beneath the surface by means of a cork-borer, and weighed about 3 gm. Each sample was put in a sterilized test-tube.

The weight of the test tubes had previously been determined, and they were now weighed once more in order to obtain the exact weight of the amount of soil that had been placed in them. When

the weight had been determined, the soil was diluted 5000 times with sterilized water. This mixture was shaken for three quarter of an hour in a shaker in order to separate the soil particles from each other and to obtain a more even distribution of the mycelium and the spores.

An amount of 1 cc of each soil suspension was mixed with 15 cc molten cherry-agar at 40° C, and this mixture was poured out into a Petri dish, where it was allowed to solidify. At this dilution the number of fungus colonies that developed in each dish, fluctuated between 15 and 30.

In order to find out which of the fungi would be able to exercise an antagonistic effect on *Thielaviopsis*, each Petri dish was inoculated at 5 different places with this fungus, and as the observation of the antagonists is often obstructed by the fast growth of other fungi, and also because the counting of the number of colonies too may become difficult in this way, the dishes were kept at the relatively low temperature of 16° C.

The Petri dishes were regularly inspected in order to estimate the total number of fungus colonies, the number of colonies with an antagonistic effect on *Thielaviopsis* and the number of *Thielaviopsis* colonies derived from the soil (Table 15).

The figure that is given in this table for the number of fungus colonies, is the total number of colonies counted in a set of 5 Petri dishes, and as the colonies in the 5 dishes were obtained from 5 cc of a 1/5000 dilution of a certain weight of moist soil, the number per gm moist soil must be 1000 times as large. The figures in the table may therefore be multiplied by 1000.

It is rather remarkable that in the cultures obtained from the infested soils of group A, i.e. of the group to which no *Thielaviopsis* suspensions were administered, no *Thielaviopsis* colonies were found. As roots of *Primula obconica* infected with *Thielaviopsis* were present in large numbers in this soil, the most plausible explanation of the absence of *Thielaviopsis* colonies in the cultures seems to be that most of the chlamydospores and endoconidia occur in and on the diseased roots, and that but very few of them are set free in the soil. Thus, the first of the four questions that were formulated above, can not yet be answered. Although the parasite could not be isolated from the diseased soil of group A, it need not be doubted that it must have been present, not only because the primulas that in the past had been grown in this soil, had become diseased, but because those that were planted in it in the period following that of these experiments, also became infected (see next section). A decrease of the amount of the inoculum could therefore not be demonstrated.

In group A as well as in group B the number of fungus colonies obtained from the "diseased" soil was about 30 % higher than that obtained from the "healthy" soil, notwithstanding the latter seemed to be a more favourable substrate as it contained more organic matter. In group B the character of the fungus flora remained almost constant, in spite of the regular addition of the *Thielaviopsis* suspension. It

TABLE 15

Number of fungus colonies counted on five agar plates each inoculated with 1 cc of a suspension containing 1 gm soil in 5 l water

	Group A without addition of *Thielaviopsis basicola*				Group B with addition of *Thielaviopsis basicola* every other day									
	"healthy" soil		"diseased" soil		"healthy" soil					"diseased" soil				
	Number of fungus colonies	Number of antagonists	Number of fungus colonies	Number of antagonists	Number of fungus colonies other than *Thielaviopsis basicola*	Number of antagonists	Number of *Thielaviopsis basicola* colonies	Total number of fungus colonies	*Thielaviopsis basicola* colonies in % of total number	Number of fungus colonies other than *Thielaviopsis basicola*	Number of antagonists	Number of *Thielaviopsis basicola* colonies	Total number of fungus colonies	*Thielaviopsis basicola* colonies in % of total number
Date of sampling	1	2	3	4	5	6	7	8	9	10	11	12	13	14
2/3	70	7	102	8	103	10	—	—	—	104	6	—	—	—
4/3	66	6	113	9	70	7	—	—	—	103	8	—	—	—
6/3	88	4	114	10	88	5	22	110	20.0	115	9	30	145	20.7
8/3	81	8	109	10	85	3	43	128	33.6	105	8	66	171	38.6
10/3	89	2	107	10	81	8	30	111	27.0	107	9	31	138	22.5
12/3	85	8	108	7	92	7	21	113	18.6	120	12	13	133	9.8
14/3	85	9	111	11	91	8	26	117	22.2	116	9	17	133	12.8
16/3	92	9	128	12	89	5	21	110	19.1	124	12	11	135	8.2
18/3	87	9	115	8	97	9	19	116	16.4	120	6	16	136	11.8
20/3	84	10	117	11	101	5	26	127	20.5	111	12	13	124	10.5
22/3	81	3	105	9	84	4	20	104	19.2	108	11	10	118	8.5
28/3	—	—	—	—	78	8	8	86	9.3	120	12	7	127	5.5
Total other than 28/3	908	75	1229	105	981	71	228	1036	—	1233	102	207	1233	—
Aver. other than 28/3	82.55	6.82	111.73	9.55	89.18	6.45	25.33	115.11	21.84	112.09	9.27	23.0	137.0	15.94

looked as if the microflora had reached a state of equilibrium which it tended to preserve against the attempts at interference. The answer to the second question is therefore that the density of the fungus population in the "diseased" as well as in the "healthy" soil was not noticeably affected by the regular introduction of new *Thielaviopsis* material.

More antagonistic colonies were isolated from the "diseased" soil than from the "healthy" soil of both groups. Therefore a stronger inhibition of *Thielaviopsis* was to be expected in the "diseased" soil. It is, on the other hand, rather unexpected that the number of colonies by which the antagonists are represented in the cultures isolated from the soil of the B group is of the same order of magnitude as the number of colonies of antagonists isolated from the soil of the A group. This means that the regular addition of *Thielaviopsis* inoculum does not lead to an increase in the density of the population formed by the antagonists. This, therefore, is the answer to the third question.

That the number of *Thielaviopsis* colonies in the cultures obtained from the soil of the B group gradually decreased, is doubtless a remarkable phenomenon; it shows that the density of the *Thielaviopsis* population which was reached after the first inoculations, could not be maintained. This decrease in density stands out more clearly when the figures in the columns 7, 9, 12 and 14 of table 15 referring to the isolations carried out in three consecutive periods are averaged, as in this way the influence of accidental differences is lessened (Table 16). This could, of course, not be done with the figures relating to the last isolations which were carried out on the 28th March, 6 days after the last infestation (22nd March).

TABLE 16

Number of *Thielaviopsis* colonies isolated from soils which from the 4th to the 22nd March were enriched every other day with suspensions of this fungus. The isolations were carried out every other day from the 6th to the 22nd March and once more on the 28th March

Isolations on	"Healthy soil"			"Diseased soil"		
	Total number of *Thielav.* colonies	Average number of *Thielav.* colonies	*Thielav.* colon. as % of total number of fungus colon.	Total number of *Thielav.* colonies	Average number of *Thielav.* colonies	*Thielav.* colon. as % of total number of fungus colon.
6.3; 8.3; 10.3	95	32	27.2	127	42	28.0
12.3; 14.3; 16.3	68	23	20.0	41	14	10.2
18.3; 20.3; 22.3	65	22	18.7	39	13	10.3
28.3	8	8	9.3	7	7	5.5

The number of *Thielaviopsis* colonies that in group B could be isolated from the diseased soil was at first somewhat higher than that which could be obtained from the healthy soil, but this lasted a short time only, and then the number that could be isolated from

the diseased soil sank to a value considerably below that obtained from the healthy one. It is noteworthy that in the cultures from the diseased soil the average number sank in the course of the first six days from 42 to 14, whereas in the healthy soil the average number sank in the same period from 32 to 23. When the number of *Thielaviopsis* colonies is expressed as a percentage of the total number of fungus colonies, it appears that this percentage is in the diseased soil about half that found in the healthy one. The higher density of the fungus population and especially the greater abundance of antagonists in the diseased soil may be responsible for this more rapid decrease of *Thielaviopsis*.

Although the number of *Thielaviopsis* colonies that could be isolated in the period 18/3–22/3 was slightly lower than that isolated in the period 12/3–16/3, the differences are so small that it seems justified to assume that a balance had been reached and that further addition of the *Thielaviopsis* suspension would have had no effect.

The fourth question therefore may be answered in this way that the number of *Thielaviopsis* colonies which can be isolated from "diseased" as well as from "healthy" soil when these soils are infested at regular intervals with this fungus, probably needs but a short time to reach a more or less constant value.

Six days after the administration of the *Thielaviopsis* suspension had been stopped, the number of colonies that could be re-isolated, showed a considerable decrease (Tables 15 and 16). The decrease was greater in the cultures obtained from the "healthy" soil (from 22 to 8) than in those obtained from the "diseased" one (from 13 to 7). The further history of the microflora of these soils was not studied.

When a micro-organism is regularly added to a soil, it is not unusual that this addition is followed by an increase in the development of its antagonists. In the investigations of which the results are recorded in this paper, it appeared that infestation of the soil with *Thielaviopsis basicola* does not cause a better development of its antagonists, but that, on the contrary, the development of *Thielaviopsis* is kept in check by the antagonists in their normal density and perhaps by the presence of other fungi which are part of the ordinary soil population.

5.2. THE PATHOGENICITY OF THIELAVIOPSIS BASICOLA IN THE SOILS OF WHICH THE MICROFLORA WAS STUDIED IN THE PRECEDING SECTION

The pots that had been used in the experiments described in the preceding section, were, after the experiments were finished, planted each with a young specimen of *Primula obconica* (height 3–4 cm) in order to test by the aid of the latter the pathogenicity of the *Thielaviopsis* population that at that moment was present. To this end the pots were kept for 104 days in a greenhouse at an average day temperature of 22° C. In each pot the pH of the soil was determined; the average values found for the 5 pots of which each set consisted, are recorded in the last column of Table 17. In the course of the

sojourn in the greenhouse the pH in the pots with the more strongly diseased plants showed a tendency to decrease, but the differences were so small that it is not probable that they would have influenced the growth of the plants.

In order to give an impression of the condition of the plants at the end of the experiment, in table 17 figures are collected with regard to the following features (1) total length of shoots, (2) total number of leaves ,(3) average length and (4) average width of leaves, (5) total number of inflorescences, (6) average length of inflorescences, (7) dry-weight of the shoots, and (8) dry-weight of the roots.

The condition of the plants that had been cultivated in the pots belonging to group A (Table 15), was as follows:

In the "healthy" soil the plants were healthy and well developed with large green leaves and a considerable number of inflorescences and flowers. The roots were healthy and well developed, white and without the symptoms of an infection by *Thielaviopsis*, abundantly ramified, long and distributed over a large area. The plants of this set were far better developed than those of the other ones.

In the "diseased" soil the plants were less well developed; the leaves were on the whole smaller, the inflorescences fewer in number, less well developed and provided with a smaller number of flowers. The roots too were less well developed and less well distributed in the soil; in many places they showed a brown discoloration and were rotten, although the top part was, as a rule, still white. In the infected parts of the roots a large amount of mycelium and of chlamydospores of *Thielaviopsis* was present.

The condition of the plants that had been grown in the pots belonging to group B (Table 15), was worse than that of the plants of group A that had been cultivated in the "diseased" soil.

The heaviest infection was found in the plants that had been grown in the "healthy" soil to which in this case at regular intervals a *Thielaviopsis* suspension had been administered. Nearly all the plants were on the verge of dying. The roots were thin and had hardly grown out, and they were almost entirely rotten and showed a brown to black discoloration.

In the "diseased" soil to which the *Thielaviopsis* suspension had regularly been administered, the plants had also badly developed, although on the whole somewhat better than those belonging to the previous set. The roots were weak and had hardly grown; for the greater part they were brown and rotten; they were moreover thin and brittle. The infected parts of the roots contained large amounts of chlamydospores. The fungus could be isolated from them. The infection was doubtless not so severe as in the preceding set.

It appears from Table 17 that the plants in the not-infested "healthy" soil showed by far the best development, and as these plants did not show a single symptom of the disease, they can be regarded as a control set.

The most severe infection was found in the two sets of plants belonging to the group B, i.e. to the plants that grew in a soil that

TABLE 17

Condition of *Primula obconica* plants after 3 months growth in "healthy" and in "deseased" soil that had been left as it was (group A) or to which several times a *Thielaviopsis* suspension had been added (group B)

	Total length of shoots in cm	Total number of leaves	Average length of leaves in cm	Average width of leaves in cm	Total number of inflorescences	Average length of inflorescences in cm	Dry-weight of shoots in gm	Dry-weight of of roots in gm	pH of the soil
	1	2	3	4	5	6	7	8	9
Group A:									
"Healthy" soil	1607.0	79	15.14	8.12	27	15.23	23.48	2.42	6.9
"Diseased" soil	651.0	51	10.32	6.14	9	13.88	9.30	1.70	6.7
Group B:									
"Healthy" soil repeatedly infested with *Thielaviopsis*	250.0	22	9.02	5.05	5	10.30	3.27	0.42	6.4
"Diseased" soil repeatedly infested with *Thielaviopsis*	311.0	27	9.90	5.40	3	14.63	4.03	0.70	6.5

had been infested at regular intervals; the plants that grew in the "healthy" soil were most strongly diseased.

To some extent the results that were obtained by the investigations with regard to the development of *Thielaviopsis* in non-sterilized soil, and that are summarized in the Tables 15 and 16, are reflected in those of the two last-mentioned sets of experiments with *Primula obconica*, as in these earlier experiments the *Thielaviopsis* population underwent in the "diseased" soil a stronger decrease in density, and reached a lower final level. Although the fungus could not be isolated from the "diseased" soil to which no suspension of *Thielaviopsis* had been administered, the plants that were grown in this soil nevertheless became severely infected, and although a direct proof of the presence of the fungus could in this case not be given, its presence could convincingly be demonstrated by the aid of a susceptible host.

6. INOCULATION EXPERIMENTS WITH NICOTIANA GLUTINOSA

6.1. Influence of physiogenic factors and of the presence of thielaviopsis in the soil on the development of nicotiana glutinosa

It is well-known that the cultivation of *Nicotiana glutinosa* in a glass-house may offer difficulties. A yellowing of the leaves and even a premature dying may occur, and sometimes the plants are weakened by an attack of *Thielaviopsis*. Unfavourable physiogenic factors and the presence of the fungus in the soil may cooperate to cause a syndrome of symptoms which is difficult to disentangle. An accurate assessment of the pathogenicity of *Thielaviopsis* is possible only when

the harm caused by unfavourable external conditions is eliminated. To this end it seemed desirable to determine first of all under what conditions a healthy development of the plants may be obtained.

As temperature, light intensity and air humidity seemed to be the factors that deserved special attention, the experiments were confined to them. The influence of each of them was tested at two levels, viz.

Temperature:	25° C (1)	or	19° C (2)
Light intensity:	sunlight (3)	or	shade (4)
Air humidity:	r.h. 95–100 (5)	or	r.h. 25–60 (6)

Combination of these conditions is possible in 8 ways, viz.

1. 3. 5.	1. 3. 6.	1. 4. 5.	1. 4. 6.
2. 3. 5.	2. 3. 6.	2. 4. 5.	2. 4. 6.

and all these combinations were tested.

The experiments were carried out in a period of 28 days, viz. from April 15th to May 12th. The average day length during this period was 14.40 hours. The plants were grown in sterilized garden soil of which the pH varied between 5.0 and 5.4, and for each of the 8 combinations of conditions two sets of 16 plants each were used, one of the sets of 16 being infested with *Thielaviopsis*, and the other set serving as control. The infestation was effected by means of spore suspensions, which were mixed as thoroughly as possible with the soil. The quantity of inoculum was approximately 3000 to 3500 infection units per cc soil; these units were partly conidia and partly chlamydospores. The choice of this quantity was based on the findings of LEVYKH (1938). The experiments were carried out with young, healthy plants, 5–7 cm in height, and for the various sets equivalent ones were chosen. The two sets that were used for each combination of conditions were placed together in a glass chamber.

In order to obtain the relative humidity of 95–100 (5) in sunlight (3), the plants were artificially misted. For the plants in the shade the same degree of humidity was obtained by clothing the glass chambers on the inside with filter-paper and keeping the latter moist, and by covering the chambers with a glass plate placed on moist filter-paper. The water content of the soil was always kept more or less at the same level. Shading was effected by means of filter-paper by which the sunlight was shut out as much as possible, without, however, causing a noticeable rise of the relative humidity. As the sky was hardly ever overcast, the circumstances were favourable for the study of the effect of sunlight.

After about 20 days the condition of the infected plants that were exposed to direct sunlight, was getting worse and worse, and after 28 days they were on the verge of dying. In order to obtain properly comparable results, the experiment should be ended before any plant had succumbed.

At the end of the experiment all the plants were appraised according to a "condition index". In the latter the figure 1 was given to the

weakest plants found in the 16 sets, and the figure 10 to the healthiest and best developed ones; the rest of the plants were awarded figures between these extremes. This mode of assessment made it possible to obtain a rather reliable picture of the differences between the various sets.

The "condition figures" assigned to each of the 16 plants of a set have been added, and these sums are for each of the 16 sets of plants set down in the graphs reproduced in Fig. 1.

The experiment shows that the condition by which the development of *Nicotiana glutinosa* is most unfavourably influenced, is the exposition

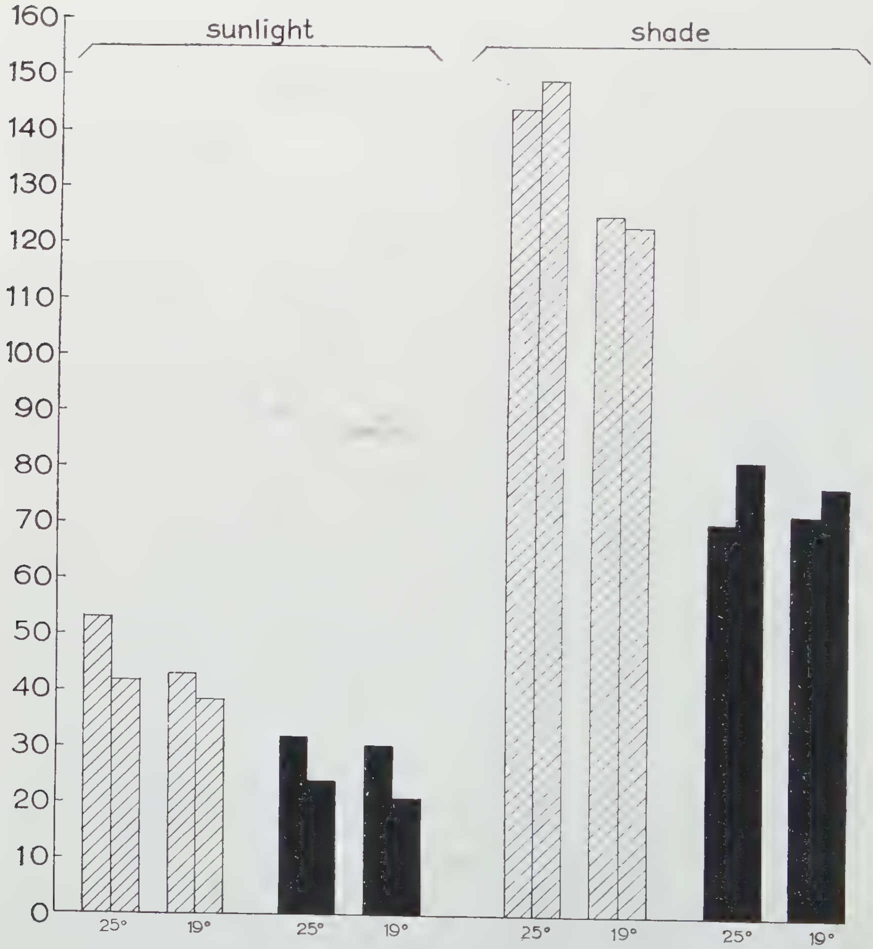

Fig. 1. Total "condition index" of *Nicotiana glutinosa* plants after 28 days of growth in infested (black) and uninfested soil (shaded), and under influence of various external factors.

The left column of each pair refers to a high humidity, the right one to a low humidity.

to full sunlight. The latter caused a poor growth; the plants were dwarfed, and flowering started at an early stage. Moreover, the leaves showed a yellow discoloration, particularly around the veins; they had therefore a mottled look. The plants nevertheless succeeded in completing their life cycle.

With regard to the influence of the temperature it should be noted that at the beginning the plants grew noticeably better at 25° C than at 19° C. However, towards the end of the experiment the difference became less pronounced. The best developed plants were those that had been grown at 25° C in the shade; the weakest ones were those that at 19° C had been exposed to sunlight and to a low relative humidity. Under the conditions of this experiment temperature may be regarded as the condition that is second in importance.

Low air humidity affected the plants only when they were grown in sunlight, and then at the higher as well as at the lower temperature. The plants that were grown in the shade, proved to be indifferent to the humidity of the air. In this experiment the last-mentioned condition seemed to be of least importance.

In the infested soil all plants proved to be attacked by the parasite, although the degree of infection varied with the combination of the physiogenic conditions. The plants grown in direct sunlight showed a syndrome that was partly caused by the physiogenic conditions and partly by the infection, whereas the plants that were grown in the shade, were damaged only by the parasite. In the infected plants the leaves, especially the lower ones, showed either a more or less even yellowing or a withering, depending upon the severity of the attack, and, in contrast to the leaves that showed a yellow discoloration because of their exposition to direct sunlight, the yellow leaves of these plants withered shortly afterwards.

The condition index of the infected plants that had been kept in the shade, was much higher than that of the corresponding plants in direct sunlight; that of the worst set in the shade was still higher than that of the best set of the not-infected plants that had been exposed to direct sunlight. However, when the condition index of the shaded plants is compared with that of the sunlight-plants, it appears that the decrease caused by the infection is in the first-mentioned group of plants greater than it is in the latter.

In the shaded plants the difference in development between the infected and the not-infected ones appeared to be larger in the sets that had been grown at a temperature of 25° C than it was in the corresponding sets that had been grown at 19° C. It appears that not only the development of *Nicotiana glutinosa* is favoured by the higher temperature, but also that of the parasite. This is in agreement with earlier findings according to which the optimum temperature for the growth of *Thielaviopsis* comes nearer to 25° C than to 19° C.

The bad condition of the plants that had developed in direct sunlight has previously led to the assumption that sunlight favours the development of black root-rot in *Nicotiana glutinosa*. This experiment, however, shows that shaded plants are actually more severely

damaged by the parasite than the plants grown in sunlight. For an accurate assessment of the pathogenicity of *Thielaviopsis* it is therefore inadvisable to grow *Nicotiana glutinosa* in direct sunlight, since the influence of the parasite is in that case obscured by that of the exposition to direct sunlight. However, under all the conditions tested in this experiment *Nicotiana glutinosa* proved to be very susceptible to the attacks of the parasite, a fact that was already mentioned by Johnson in 1916.

The different conditions under which *Nicotiana glutinosa* was grown, appeared to have no influence on the development of the root system. Differences in the development of the latter were always due to the presence or absence of the parasite. In the not-infected plants the roots always were healthy, white and more or less evenly distributed through the soil. The roots of the infected plants, on the other hand, were poorly developed and spread but imperfectly in the soil; they were mostly brown, and several of them were dead and rotten. Occasionally the roots appeared to be somewhat better developed, but even then their development did not correspond to that of the aerial parts. In the diseased roots the chlamydospores of *Thielaviopsis* were present in large amounts; from these roots the fungus could be isolated.

When the infected plants that had been grown in the shade, either at a temperature of 19° C or at 25° C, were exposed to sunlight and to a somewhat drier air, they wilted within ten minutes, whereas the not-infected plants remained fresh, even when the exposure to sunlight and drier air was extended to one hour. This points to a retardation either of the water uptake or of the water transport in the infected plants, a retardation for which *Thielaviopsis* is very likely responsible. Toxic substances secreted by the parasite might be directly involved.

The response of *Nicotiana glutinosa* to the two temperatures tested in this experiment is not the same as that found by other investigators in *Nicotiana tabacum*. In the latter the temperature exercised a stronger influence on the degree of infection. However, it is not excluded that the influence of the temperature on the infection of *Nicotiana glutinosa* would have been more marked when the amount of *Thielaviopsis* inoculum had been smaller.

The pH of the soil varied, as stated above, between 5.10 and 5.40, but as this must be regarded as a rather narrow range, and as the variation, moreover, was not related to differences in the external conditions, it may be assumed that it did not affect the development of the host plants nor that of the parasite. For *Nicotiana tabacum*, however, the significance of the pH of the soil for the development of black root-rot has been recognized more than once and by various investigators.

In all the combinations of light, temperature and air humidity that were tested in this experiment, the disease developed at a pH lower than 5.5, whereas according to Doran (1929) and others in *Nicotiana tabacum* black root-rot is never met with at a pH lower

than 5.6. This difference may, however, partly or entirely be due to the fact that in the experiment with *Nicotiana glutinosa* a much larger amount of *Thielaviopsis* inoculum was used than in Doran's experiments with *Nicotiana tabacum*. In this connection it should be remembered that according to Johnson and Hartman (1919) the resistance of acid soils against the infection with *Thielaviopsis* may be overcome completely, at least if a susceptible variety of *Nicotiana tabacum* is used, by increasing the amount of inoculum.

In another section of the greenhouse in which the experiment with *Nicotiana glutinosa* was carried out, more plants of this species and also plants of *Nicotiana tabacum* were grown for other purposes. These plants grew in the same soil as the plants that were used for the experiment, i.e. at a pH lower than 5.6, but this soil had not been sterilized and was naturally infested with *Thielaviopsis*. The temperature in this section was approximately 25° C, and the *Nicotiana glutinosa* plants were protected from the direct sunlight, those of *Nicotiana tabacum* not. In the latter no signs of black root-rot were ever observed, and this is in good agreement with the experiences of other investigators, but in nearly all the plants of *Nicotiana glutinosa* the infection was clearly discernible, especially during the later stages of their development. This observation therefore confirms the view that in *Nicotiana glutinosa* a higher temperature and a pH lower than 5.6 do not control the black root-rot to the same extent as they do in *Nicotiana tabacum*.

6.2. Influence of some antagonists upon the pathogenicity of thielaviopsis basicola

The experiment described in the preceding section showed that for an assessment of the pathogenicity of *Thielaviopsis basicola* by means of *Nicotiana glutinosa* the latter should not be grown in direct sunlight, as in that case the syndrome caused by this mode of cultivation may obscure the symptoms of the disease. The experiment also gave indications with regard to the influence of temperature and air humidity, and on account of these indications the following combination of conditions was chosen to carry out some investigations on the influence exercised by antagonists on the pathogenicity of *Thielaviopsis*: a temperature of 25° C, shade and a high humidity. The fungi whose antagonistic activity was tested, were *Penicillium spiculisporum*, *Penicillium expansum*, *Aspergillus fumigatus* and *Penicillium spinulosum*.

For this investigation the following sets of plants were used:

A. Plants grown *in sterilized soil* with a pH of 5.52–5.98
- *a.* Soil not infested
- *b.* ,, infested with *Thielaviopsis*
- *c.* ,, ,, ,, *Thielaviopsis* + *Penicillium spiculisporum*
- *d.* ,, ,, ,, *Penicillium spiculisporum*
- *e.* ,, ,, ,, *Thielaviopsis* + *Penicillium expansum*

f. ,, ,, ,, *Penicillium expansum*
g. ,, ,, ,, *Thielaviopsis* + *Aspergillus fumigatus*
h. Soil infested with *Aspergillus fumigatus*
i. ,, ,, ,, *Thielaviopsis* + *Penicillium spinulosum*
j. ,, ,, ,, *Penicillium spinulosum*

B. Plants grown *in unsterilized soil*
k. Soil infested with *Thielaviopsis* (pH 6.65)
l. ,, not infested (pH 6.16).

The sets ***a***, ***b*** and ***l*** served as controls.

The number of plants per set was 20; it were young, vigorously growing plants with a height of 6–8 cm, and they were distributed in such a way over the sets that the latter were all of similar composition.

The infection was carried out in the same way as in the experiment described in the preceding section, i.e. by means of spore suspensions. Where *Thielaviopsis* was used in conjunction with an antagonist, the two spore suspensions were thoroughly mixed before they were added to the soil. The amount of *Thielaviopsis* inoculum was the same as in the previous experiment, viz. 3000–3500 infection units per cc soil. Of the antagonist 3000–5000 spores per cc soil were used.

The experiments were carried out in the period May 28th to June 17th, and the average day length in this period was 16 hours 29 minutes.

In contrast with the 28 days of the previous experiment, this set of experiments lasted but 20 days. At the end of this period the plants were already further developed then in the corresponding sets of the previous experiment and the symptoms of the disease were much more pronounced. It seems plausible to assume that the development required one third less time. The cause of this more rapid development of the host as well as of the symptoms of the disease can not be found in differences in temperature, light intensity, air humidity, soil features or the amount of inoculum, as all these conditions were the same. The plants with which this set of experiments were started, were somewhat larger, but this difference can hardly have been of importance. The pH of the soil was approximately 0.5 higher, and this may have favoured the development of the parasite, but hardly that of the host. The average day length was 1 hour 49 minutes longer, and it is not impossible that this factor may have been responsible for a more rapid growth. Stover (1950b) is of opinion that the average day length is of importance for the pathogenicity of *Thielaviopsis* in tobacco plants. According to him a minimum day length of 12 hours is required.

After approximately 10 days the plants in the sterilized soil that had been infested with *Thielaviopsis* alone, appeared to be heavily attacked. They showed the symptoms of the disease, and lagged far behind the control plants. At this stage there was as yet hardly any

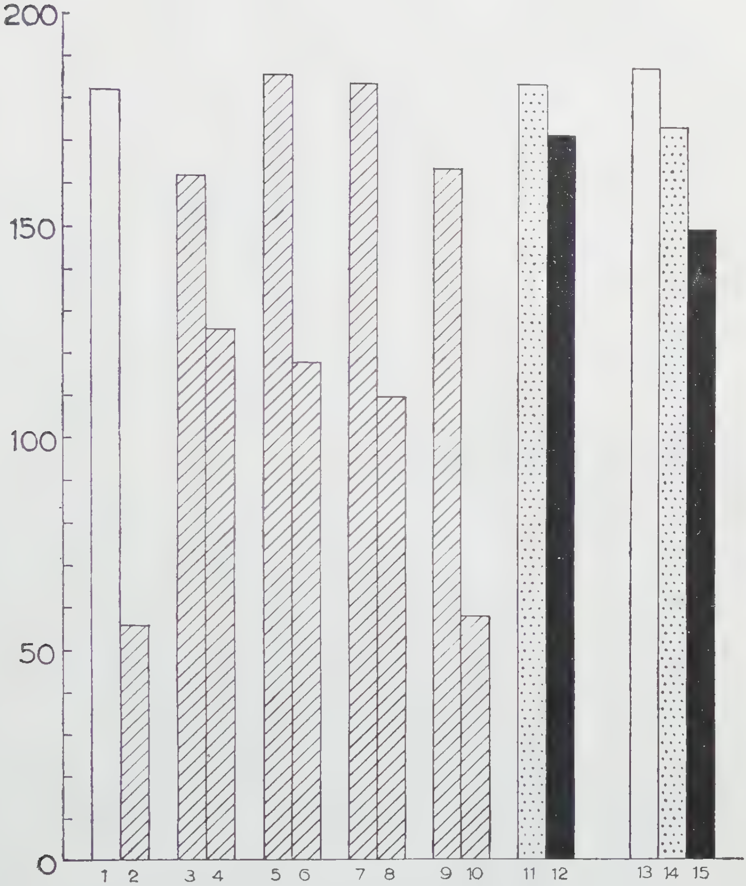

Fig. 2. "Condition index" of *Nicotiana glutinosa* plants after 20 days growth (1–12) and after 35 days growth (13–15) in sterilized soil (1–10 and 13) and in unsterilized soil (11, 12, 14 and 15).

1 = not infested
2 = infested with *Thielaviopsis basicola*
3 = ,, ,, *Penicillium expansum*
4 = ,, ,, *Thielaviopsis* + *Penicillium expansum*
5 = ,, ,, *Penicillium spiculisporum*
6 = ,, ,, *Thielaviopsis* + *Penicillium spiculisporum*
7 = ,, ,, *Penicillium spinulosum*
8 = ,, ,, *Thielaviopsis* + *Penicillium spinulosum*
9 = ,, ,, *Aspergillus fumigatus*
10 = ,, ,, *Thielaviopsis* + *Aspergillus fumigatus*
11 = unsterilized soil uninfested
12 = unsterilized soil infested with *Thielaviopsis*
13 14 and 15 = same as 1, 11 and 12 but assessed 15 days later.

difference between the other sets of plants, with the exception of that in the soil which had been inoculated with the mixture of *Thielaviopsis* and *Aspergillus fumigatus*. It looked therefore as if the antagonists had been able to keep the infection with *Thielaviopsis* in check. However, in the course of the next 10 days the plants of the sets growing in soil that had been inoculated with a mixture of *Thielaviopsis* and one of the three *Penicillium* species became also distinctly infected by the parasite, although not to the same degree as the plants in the sterilized soil that had been infested with *Thielaviopsis* alone and in that which had been inoculated with the mixture of the latter and *Aspergillus fumigatus*; these plants were on the verge of dying.

On the 20th day the experiment was ended, and the plants of each set were assessed according to a "condition-index" similar to that used in the previous experiment (Fig. 2). With the two sets growing in the unsterilized soil (11 and 12), in which the plants showed hardly any symptoms of the disease, and with the set in the sterilized soil that had not been infested with *Thielaviopsis* (1), the experiment was continued for another fortnight.

Penicillium expansum, *Penicillium spiculisporum* and *Penicillium spinulosum* appeared to exert an inhibiting effect on the pathogenicity of *Thielaviopsis*, but *Aspergillus fumigatus* failed to do so. This fungus and *Penicillium expansum* adversely affected the development of *Nicotiana glutinosa*, while *Penicillium spiculisporum* and *Penicillium spinulosum* hardly exerted any influence.

In the unsterilized soil the natural microflora appeared to have exerted a strong inhibiting effect on the pathogenicity of *Thielaviopsis*, despite the fact that the soil was severely infected with the latter and in spite of the addition of a suspension of its spores.

At the end of the experiment the control plants in the unsterilized soil (11) looked, despite the natural infestation of the soil with *Thielaviopsis*, better than the control plants in the sterilized soil that had not been inoculated with this fungus (1). In unsterilized soil that had been inoculated with *Thielaviopsis* (12), *Nicotiana glutinosa* grew better than it did in the sterilized soil that had been inoculated with a non-parasitic soil fungus such as *Penicillium expansum* (3) or *Aspergillus fumigatus* (9).

The final condition-index of the plants belonging to the sets with which the experiment was continued for a fortnight (1, 11 and 12) is given also in Fig. 2 (13, 14 and 15).

The plants in the sterilized soil (13) showed the highest index, directly followed by those in the unsterilized soil. In the set growing in the unsterilized soil to which *Thielaviopsis* had been added, the index appeared to have decreased in this fortnight (12 and 15), but it was still higher than that which had been found in the sets grown in sterilized soil to which the mixture of the spores of *Thielaviopsis* and of one of the antagonists had been administered (4, 6, 8 and 10). Even *Penicillium expansum* (4) appeared to have a less strongly inhibiting effect than the natural microflora of this garden soil.

If the assessment in all sets had been postponed till the end of the

extra fortnight, the difference between the plants in the sterilized soil to which *Thielaviopsis* had been added and those in the unsterilized one which had been inoculated with this fungus would have been much greater, since the development of the plants in the former lagged, throughout the whole period, behind that of the plants in the latter.

In the various sets that were grown in sterilized soil the condition of the roots proved to correspond more or less with that of the aerial parts. In the soil that had not been infested with *Thielaviopsis* (1), the roots were healthy and well developed. In the soil that had been inoculated with *Thielaviopsis* alone (2), the roots were severely infected; they ranged from poorly developed to completely shrivelled, and almost all were brown and had rotten. More or less the same condition was found in the roots that had developed in the soil which had been inoculated with the mixture of *Thielaviopsis* and *Aspergillus fumigatus* (10). In the soil that had been infested with the mixture of *Thielaviopsis* and *Penicillium spiculisporum* (6) and in that where the infestation had been performed with the mixture of *Thielaviopsis* and *Penicillium expansum* (4), the roots proved to be affected by the parasite, and an intensive brown discoloration and rotting had taken place, but their condition was nevertheless much better, ranging from adequate to good, and they were less severely diseased than those of the plants in the soil inoculated with *Thielaviopsis* without an accompanying antagonist (2). In the soil that had been inoculated with the mixture of *Thielaviopsis* and *Penicillium spinulosum* (8) the roots were considerably more infected and worse developed than in the sets 4 and 6, in which the two other *Penicillium* species had been used as antagonists, but they were better developed than in the sets 2 and 10, i.e. in the soil that had been inoculated with *Thielaviopsis* without the addition of an antagonist and in that in which *Aspergillus fumigatus* had to play the part of the antagonist. In the other sets the roots were healthy and well developed, although there were small differences, those in the soil that had been inoculated with *Aspergillus fumigatus* (9) and those in that with *Penicillium expansum* (3) being somewhat less well developed.

In the unsterilized soil the roots of the control plants (14) showed an extensive brown discoloration accompanied by rotting, but they were nevertheless well developed, and at the base of the stem many new and healthy roots were growing out. In the set that developed in the soil that had been inoculated with *Thielaviopsis* (12), the roots were far more strongly infected and more poorly developed.

In the sterilized soil the pH varied between 5.52 and 5.98, and, as in the experiment described in the preceding section, there appeared to be no correlation with the condition of the *Nicotiana glutinosa* plants. This pH was higher than in the previous experiment, where it varied between 5.10 and 5.40, and its range partly overlapped the "critical region" (pH 5.6–5.9) in which, according to ANDERSON, OSMUN and DORAN (1926) and DORAN (1929), black root-rot is almost certain to cause trouble. In the unsterilized soil the pH was even higher, viz. 6.15 and 6.65, and here the plants were nevertheless not

so strongly affected as in the sterilized soil with the lower pH. It appears therefore that the natural microflora of the soil exerted a more strongly inhibiting effect upon the pathogenicity of *Thielaviopsis* than the antagonists individually did in a soil with a pH that was less favourable to the development of the parasite.

The experiments with four different antagonists described in this section revealed that *Penicillium expansum* exercised the strongest antagonistic effect on *Thielaviopsis*, and that it is followed by *Penicillium spiculisporum* and *Penicillium spinulosum*. This result is in agreement with that obtained by the experiments in vitro (Table 4). The natural microflora occurring in a garden soil may exercise an even more important influence on the pathogenicity of *Thielaviopsis basicola*. As this root parasite is generally present in garden soil, it obviously does not disappear from the latter under the influence of other micro-organisms; however, it may be kept in check by the latter.

SUMMARY

That the damage which *Thielaviopsis basicola* (Berk. et Br.) Ferraris may cause to a definite host plant, is not always equally severe, has been known already for a long time. This variability has usually been ascribed to physical factors operating in the soil, but another circumstance might also be of importance, viz. the presence of micro-organisms acting as antagonists. It is rather striking that the influence which such organisms might exercise on *Thielaviopsis basicola*, has received so far little or no attention, and for this reason a study of this problem in vitro as well as in the soil seemed appropriate.

Thielaviopsis basicola was isolated from the roots of *Primula obconica* as well as from those of *Nicotiana glutinosa*, and by using the isolation method of Yarwood (1946) it was found that at Baarn (Netherlands) this root parasite is very common in garden soil.

In order to obtain antagonists, samples of various kinds of soil were plated out, and the fungi that developed on the plates, were isolated and tested as to their power to inhibit the growth of *Thielaviopsis*. On cherry agar about 38 of them showed an antagonistic effect. In further experiments the antagonistic activity of these fungi was estimated by means of filtrates obtained from cultures in cherry juice. In this way about 20 % of the 38 fungi were found to cause in *Thielaviopsis* a notable growth inhibition. The strongest antagonistic activity was found in the culture filtrates of *Aspergillus fumigatus* Fres., *Penicillium expansum* (Link) Thom, *Penicillium spinulosum* (Link) Thom, *Penicillium spiculisporum* Lehman and *Penicillium roqueforti* Thom. The two last-mentioned species were so far unknown as producers of antibiotic substances.

The composition of the culture medium in which the fungi were grown, appeared to exercise a marked influence on the antibiotic activity of the culture filtrates as observed in cultures of *Thielaviopsis*. From a Czapek–Dox medium, in most instances, a more active filtrate was obtained than from a cherry-juice medium or from a culture in potato extract. In the Czapek medium, moreover, the carbon source proved to be of importance; in the case of *Penicillium roqueforti* saccharose gave the most active filtrate, whereas with *Penicillium spiculisporum* glucose and maltose proved to be more suitable. It was found, moreover, that in the case of *Penicillium roqueforti* the activity of the filtrates increased when the concentration of the saccharose in the Czapek medium was raised from 1 % to 5 %. The antibiotic activity of the filtrate of this fungus appeared to decrease when corn steep liquor was present.

The growth-inhibiting substances in the culture filtrates, with the exception

of those present in the filtrate of *Aspergillus fumigatus*, proved to be able to withstand a temperature of 103° C for 10 minutes; at room temperature they retained their activity for at least 45 days.

Penicllium roqueforti proved to produce its growth-inhibiting substance(s) mainly in the period of active growth, i.e. during the first 15 days. When the fungus was kept for more than 30 days on the same medium, the antibiotic activity of the filtrate decreased.

Different strains of *Penicillium roqueforti* were found to differ as to the inhibiting effect of their culture filtrates, whereas different strains of *Thielaviopsis basicola* reacted more or less in the same way on the culture filtrate of this antagonist.

The relation between the natural microflora of different soils and the development of *Thielaviopsis* in the latter were also studied. To this end the soils were periodically inoculated with a suspension obtained from a *Thielaviopsis* culture. Two different soils were tested, viz. a "diseased" soil, i.e. a soil in which diseased plants of *Primula obconica* had grown, and a "healthy" soil, i.e. a soil in which the Primulas had remained healthy. In the "diseased" soil, at the beginning of the experiment, the presence of *Thielaviopsis* could not be demonstrated by means of the plate method; that it nevertheless was present, follows from the fact that Primulas which were subsequently planted in this soil also became infected. Suspensions containing the mycelium and the spores of *Thielaviopsis* were added every other day, from the 4th March to the 22nd March, and every time before a new dose was given, a soil sample was taken in which the composition of the microflora was quantitatively determined. It appeared that the frequency of the soil fungi was not influenced by the periodic inoculations with *Thielaviopsis*. Contrary to the expectation, the frequency of the antagonists too remained the same.

During the first 6 days of the experiment the number of *Thielaviopsis* colonies remained high, but then a sharp decrease was noted. It was largest in the soil in which the microflora was best developed, i.e. in the "diseased" soil. During the later part of the experiment the lower level was maintained. Shortly after the additions of the *Thielaviopsis* suspension were stopped, the number of *Thielaviopsis* colonies in the soil samples decreased with 50 to 60 %.

When the experiment was ended, the soil was planted with *Primula obconica*, and then it appeared that the infection of the latter was heaviest in the "healthy" soil that had periodically been inoculated with *Thielaviopsis*. This is in agreement with the finding that the number of *Thielaviopsis* colonies that could be isolated from the "diseased" soil underwent a stronger decrease than the number that could be isolated from the "healthy" soil. The experiment with the Primulas is also of interest because it shows that the presence of a parasite can sometimes be demonstrated by means of a susceptible host where attempts to isolate it by means of the plate method remain unsuccessful.

In the experiments on the influence exercised by various antagonists on the pathogenicity of *Thielaviopsis*, *Nicotiana glutinosa* was used as a test plant. It is very susceptible to infection by *Thielaviopsis*.

First the influence exercised by various external conditions on the growth of *Nicotiana glutinosa* and on its infection by *Thielaviopsis* was investigated in order to find a suitable combination of conditions for the cultivation of this plant, i.e. a combination that does not cause a syndrome by which the symptoms of the disease might be obscured. It appeared that in direct sunlight the plants grew slowly and remained stunted; the leaves showed a yellow discoloration, and flowering started at an early stage. These symptoms may easily be mistaken for those caused by an infection with *Thielaviopsis*. A temperature of 25° C proved to be slightly better than a temperature of 19° C, but the humidity of the atmosphere had hardly any effect.

The degree to which the roots were infected, showed little or no correlation with the external conditions, but this did not apply to the plant as a whole, for the infected plants that had developed in direct sunlight were at the end of the experiment at the verge of death, whereas the infected plants that were grown in the shade, though they had lost a good deal of their vigour, were still in a fairly good condition. The "condition-index" calculated for the two groups shows, on the other hand, that the decrease in vigour shown by the shaded plants was proportionally larger than that of the sunlight plants (Fig. 1).

The amount of inoculum that is required for a severe infection of *Nicotiana glutinosa*, proved to be 3000–3500 infection units (chlamydospores and conidia) per cc soil. A dose of this strength was used for the assessment of the pathogenicity of this root parasite in sterilized soils to which an antagonist had been added, and also in an unsterilized soil, i.e. in a soil in which the natural microflora still was present. The conditions under which this experiment was carried out, were shade, a temperature of 25° C and a high relative humidity, because this are the circumstances which proved to be not only favourable for the development of the plants, but which also allowed an accurate assessment of the severity of the disease symptoms caused by *Thielaviopsis basicola* in the presence or absence of antagonists.

In sterilized soil the infection of *Nicotiana glutinosa* by *Thielaviopsis* proved to be inhibited to some extent when the soil was at the same time infested with *Penicillium expansum*, *Penicillium spiculisporum* or *Penicillium spinulosum*. *Aspergillus fumigatus* had no effect. The experiment with unsterilized soil showed that the inhibition exercised by the microflora which is normally present in the soil, is far more effective than that which could be obtained in a sterilized soil by inoculation with one of the above-mentioned antagonists (Fig. 2). Of all the antagonists tested, the three *Penicillia* proved to be most effective against *Thielaviopsis basicola* in the soil as well as in vitro.

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STUDIES ON THE INCREASED RESPIRATION OF POTATO-TUBER TISSUE AFTER INFECTION WITH GIBBERELLA SAUBINETII (MONT.) SACC.

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CONTENTS

CHAPTER I

GENERAL INTRODUCTION

It has been known for many years that structural and physiological changes take place in plants as a result of an infection with a parasitic micro-organism. These changes are markedly different dependent on the host-parasite combination. The course of an infectional disease is determined by the influence of the parasite on the plant and the reaction of the host to the attack. GÄUMANN (1946) extensively discussed this subject. More recent reviews have been published by ALLEN (1954) and CHEVAUGEON (1957).

From the literature it appears that the physiological changes in the host cells may be of various kinds and are not limited to the tissue invaded by the pathogen. In several objects an increased temperature was observed in tissue at some distance from the centre of infection (EGLITS 1933, FISCHER 1950 and YARWOOD 1953). THATCHER (1939, 1942) investigated changes in the osmotic value and in the permeability of the host cells. An increase in the amount of growth-promoting substances in infected tissue was demonstrated by DALY and INMAN (1958) and by SHAW and HAWKINS (1958). Like KIRALY and FARKAS (1957) the latter authors reported a higher activity of certain enzymes after the infection. It appeared that the chemical composition of the host cells may show a great change. ALLEN (1954) mentions changes in nitrogen-containing compounds, lipids and carotenoids, carbohydrates and phosphate. Transpiration, photosynthesis and respiration may be affected too. In general it may be said that, when a parasitic micro-organism penetrates into a plant, the metabolism of the host cells is disturbed.

The increase of the respiration rate after infection is the subject of this investigation. To explain this phenomenon an attempt may be made to detect an agent excreted by the pathogen. However, DIMMOND and WAGGONER (1953) rightly point out the necessity that the toxins demonstrated *in vitro* should be proved to be active *in vivo* too. These authors distinguish between toxins and vivotoxins. With

Gibberella saubinetii (*Mont.*) *Sacc.* as a pathogen HELLINGA (1942) reported an increased O_2-uptake by disks of potato-tuber tissue after addition of extracts from infected tissue, from mycelium or from the culture-solution in which the fungus had been grown, but it was not demonstrated that the "active principle" which was found was active *in vivo*.

It may be asked whether a respiratory increase in the host cells is only due to compounds excreted by the parasite, or whether it may be partly or wholly attributed to a change in the metabolism of the host which is independent of these compounds (wound reaction, reaction to dying cells). Therefore it seemed desirable to investigate, what happens in the host cells, and in this way to try to find an explanation for the respiratory increase. After this it may be possible to explain the rôle played by the parasite.

With the potato tuber (*Solanum tuberosum L.*) as host and *Gibberella saubinetii* (*Mont.*) *Sacc.* as a pathogen it will be studied, whether a respiratory increase can be demonstrated in the host cells after infection of the tissue, and whether this increase is accompanied by a qualitative change in the respiratory pathway. In the second place an attempt will be made to obtain information concerning the mechanism by which the respiration rate is regulated.

CHAPTER II

RESPIRATORY INCREASE IN THE HOST CELLS

1. INTRODUCTION

For many host-parasite combinations the CO_2-production and the O_2-uptake of the host-parasite complex or one of them were found to be greater than the corresponding values found in healthy tissue (ALLEN 1954, CHEVAUGEON 1957). However, owing to the often high metabolic activity of micro-organisms, this might be attributed to the respiration of the pathogen. Indeed, MARESQUELLE (1928, 1930) reported in rust-infected plant parts a strict localization of the increased O_2-uptake to the places where the parasite was present. From this observation the author concluded that a higher respiration rate in the host cells is out of the question.

After infection with *Gibberella saubinetii* (*Mont.*) *Sacc.* cylinders of potato-tuber tissue developed a greater respiration rate (HELLINGA 1942). This was attributed to an increased O_2-uptake by the potato tissue, but experiments showing the extent to which the metabolism of the fungus was responsible for the accelerated gas-exchange were not described.

First of all it is necessary to separate the metabolism of host and parasite. How far this is possible, is determined by the nature of the micro-organism concerned, by the way in which it grows on the host tissue, and by the properties of the tissue attacked. The comparison of the respiration rate of infected tissue with that of the parasite cultured on synthetic media (FISCHER 1950), has the drawback that

the growing-conditions for the parasite on the host are quite different from those which it finds in nutritional solutions. From the ecto-parasite *Erysiphe graminis var. hordeï* growing on barley leaves only the haustoria remain in the leaves when the mycelium is brushed off. Still the O_2-uptake and the CO_2-production of these leaves are distinctly greater than they are in healthy leaves (MILLERD and SCOTT 1956). ALLEN and GODDARD (1938), comparing the respiration rate of *Erysiphe*-infected wheat leaves with that of the carefully isolated epidermides of leaves with or without parasite, ascertained that the greater part of the extra O_2-uptake is to be attributed to the metabolism of the mesophyll cells of the leaves.

If it is impossible to remove the parasite from the host, an attempt may be made to find out whether in infected plant parts a reaction occurs in the tissue not invaded by the pathogen. A higher CO_2-production from cells next to the centre of infection is reported in potato tubers infected with *Bacillus phytophthorus* (EGLITS 1933). In tubers of sweet potato (AKAZAWA and URITANI 1956) and of white potato (AKAZAWA 1956a) infected with *Ceratostomella fimbriata* it is the same with the O_2-uptake of the tissue adjacent to the areas invaded by the pathogen.

As *Gibberella saubinetii* (*Mont.*) *Sacc.* growing on potato-tuber tissue penetrates only the outer cell layers and slowly spreads inward with simultaneous collapse of the superficial cells, the respiration of the tissue next to the parts invaded by the fungus can be investigated.

2. MATERIAL AND METHODS

a. *Material*

Potato tubers var. Bintje were bought on the market and stored in a cool room. *Gibberella saubinetii* (*Mont.*) *Sacc.* was obtained from the Centraal Bureau voor Schimmelcultures at Baarn and grown in culture tubes on oat meal agar.

b. *Inoculation and incubation*

Sound tubers were brushed in tapwater, externally sterilized in 4 % formalin during 2 min., and washed in running tapwater for 15 min., after which the tubers were cut by means of a sterilized knife into two halves, the cut running perpendicular to the longitudinal axis. These halves, one of which was inoculated over the whole surface of the cut with a suspension of spores in distilled water, were each transferred to a sterile glass jar (volume 0.5 L.) and placed at 25° C. Unless otherwise stated, the experiments were carried out after 5–10 days incubation.

c. *The preparation of tissue disks*

With the help of the apparatus drawn in Fig. 1 disks 1 mm. thick and 6 mm. in diameter were cut from the rather homogeneous tissue of the tubers found inside

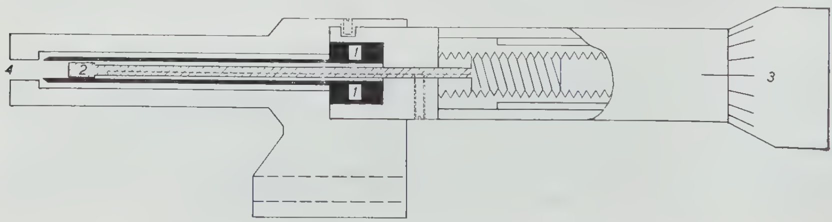

Fig. 1. Apparatus for the preparation of tissue disks.

the ring of vascular bundles. All parts of the apparatus which came into contact with the tissue, were of stainless steel. With the cork-borer (1) a tissue cylinder was bored out, which after the borer had been arrested in the apparatus, was pressed out by means of a rod (2), which was moved by turning a knob (3) provided with a scale graduated up to 0.1 mm. To prevent torsion tension in the tissue, the rod itself was not turned round. The disks were cut off along the opening of the apparatus (4) with a sterile razor blade.

From the healthy and the infected tuber halves some tissue cylinders were successively bored out perpendicular to the surface[1]) and divided into disks. From each tuber half the disks cut at the same distance from the surface were put together as tissue samples. The zonation in the infected halves was found to be parallel to the surface which was hollowed out by the fungus. The parts of the tissue cylinders which proved to contain mycelium and which showed discolorations, varied but little in extent within a tuber half, and these parts were removed. The disks from the first zone that proved to be macroscopically indistinguishable from healthy tissue, were used as the sample nearest to the area invaded by the fungus. Within each sample the variation in distance to the surface was less than 1 mm.

d. *Measurement of respiration rate*

Respiration rates, as indicated by oxygen uptake, were determined by the Warburg manometric technique, using flasks of about 15 ml. with a centre well and a side arm. They are expressed in $\mu l.O_2$ absorbed per hour per gram of tissue (fresh weight; determined before the Warburg estimation). Unless stated otherwise in the text, each flask contained 10 disks submersed in 1.5 ml. distilled water, and in the centre well 0.2 ml. KOH 10 % and a piece of filter paper (2 × 2 cm.) folded fanwise. When the CO_2-production was also to be determined, the KOH was omitted. The temperature of the waterbath was 25° C. The flasks were shaken at 130 oscillations per minute through a stroke of 4 cm. After 30 min. equilibration readings were taken at 1 hour intervals.

Preliminary experiments showed that a considerable O_2-uptake can be measured using the method described above without the experimental conditions becoming rate-limiting. Although in preparing the disks a full sterility was not pursued, interference by infections was never observed.

As there are indications that alterations in the metabolism have developed after a 24 hours' washing-period (STEWARD and STREET 1946, SCHADE and LEVY 1949, THIMANN, YOCHUM and HACKETT 1954, HACKETT and HAAS 1958, GRIFFITHS and HACKETT 1957, LOUGHMAN 1957, CALO and VARNER 1957), the disks were washed in distilled water a few times only and used practically immediately after cutting. The O_2-uptake of such disks appeared to increase gradually during the experimental period.

e. *Test for the presence of mycelium*

In order to be sure that only tissue not penetrated by the fungus was used, series of disks from infected tuber halves were laid out on moistened filter paper in petri dishes at 25° C. Mycelial development was observed only on macroscopically discolorated tissue that had been cut at the most 2–3 mm. from the surface. This is in accordance with observations made on microtome sections of infected potato-tuber tissue (Richter, unpublished) according to which, dependent on the infection-period (3–11 days), the mycelium occurred up to a distance of 1.3–3.2 mm. from the surface. So it may be concluded that the fungus does not penetrate into the intact tissue to a greater depth than about 3 mm.

Disks that are macroscopically indistinguishable from healthy tissue, do not contain mycelium. In a number of experiments all disks were laid out on moistened filter paper at 25° C. after measurement of the respiration rates. Mycelial development was never observed on these disks.

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[1]) The surface of the cut by which the tuber was divided into two halves, is always called "the surface".

3. Results and discussion

Preliminary experiments showed that, when a great number of disks were cut from the central part of a potato tuber and divided at random into samples of 10 disks, the differences between the amounts of oxygen absorbed per hour per gram (fresh weight) were small and did not exceed 10 %.

To calculate the extent of a change in respiration rate which might occur after fungal infection, one may compare the O_2-consumption of samples from the infected tuber half with that of tissue from the non-infected half of the same tuber. However, the respiration intensity of the tissue from non-infected tuber halves which had been stored at 25° C. for some days, appeared to be dependent on the place from which the disks were cut. As shown in table 1, the tissue close below

TABLE 1. μl. O_2-uptake/hour/gram (fresh weight) of samples cut at various distances from the surface of the non-infected half of two tubers. Tuber halves stored at 25° C. for 18 days. O_2-uptake average over 2 hours.

mm. from the surface	μl. O_2-uptake I	II
½–1½	100.1	97.7
1½–2½	59.1	59.2
2½–3½	55.7	57.0
3½–4½	51.4	55.9
4½–5½	48.8	55.2
5½–6½	52.7	54.9

the cutting-surface which was blocked by a cork layer, absorbed 80–90 % more oxygen than the adjacent tissue. The zone with increased respiration did not extend beyond a line 2–3 mm. from the surface. The differences between the O_2-consumption of the other samples did not exceed the variation in respiration intensity of parallel samples from an untreated tuber. The average O_2-uptake of these samples may be considered to represent the normal respiration rate of healthy tissue.

An attempt may also be made to determine the normal respiration rate with tissue from the infected tuber half itself, and to calculate the extent of the changes in respiration intensity by comparison with this rate of O_2-uptake. Assuming that the influence of the parasitically growing fungus on the metabolism of the host cells decreases when the distance to the zone with mycelium increases, it may be expected that at a certain distance from this area no change in the respiration rate is caused by the disease.

In table 2 the results are given of a representative experiment during which the O_2-consumption was determined in a number of samples taken from successive zones out of the infected half and of the non-infected half of a tuber. The respiration was distinctly accelerated near the area invaded by the fungus, and decreased with increasing distance to this area, to reach a nearly constant level in the zones at greater distance. In this experiment this level of O_2-uptake appeared to be higher than in the non-infected half of the

same tuber, but in other experiments this level was often lower than or differed but slightly from the respiration rate of healthy tissue.

In preliminary experiments with non-infected tuber halves after storage at 25° C. in a number of tubers similar differences were observed between the normal respiration rate of the tissue from the two halves. Averaged for 20 successive experiments the O_2-uptake

TABLE 2. μl. O_2-uptake/hour/gram (fresh weight) of samples from the non-infected and the infected halves of a tuber, incubated after infection at 25° C. during 10 days. O_2-uptake average over 3 hours. Dry weight estimated after heating to 100° C. overnight.

mm. from the surface	non-infected tuber half				infected tuber half				
	μl. O_2	fresh weight mg.	dry weight mg.	water content %	μl. O_2	%	fresh weight mg.	dry weight mg.	water content %
6–8	50.3	328	33	90	122.7	192	286	27	91
8–10	48.0	325	31	90	86.7	135	294	28	90
10–12	50.3	324	32	90	70.1	110	281	27	90
12–14	49.5	327	33	90	65.2		299	29	90
14–16	51.8	326	34	89	63.1		290	30	89
16–18					61.6	100	294	33	89
18–20					66.0		294	33	89

of tissue from the non-infected half and that of tissue from the area of the infected half which was supposed to be unaffected by the disease, was the same, *viz.* 58.8 μl. and 58.7 μl. O_2/hr./gram respectively. The conclusion may be drawn that the nearly constant level of O_2-uptake of samples from the infected tuber halves taken at greater distance from the surface represents the respiration rate which had not been altered by the disease.

In the experiments the extent of the respiratory increase was calculated by comparing the O_2-absorption with the average respiration rate of some samples cut from the same tuber half, but from the area which had not been affected by the fungal infection, as appeared from the nearly constant level of respiration (table 2). In addition the O_2-uptake of some samples from the non-infected half of the tuber was determined.

Table 2 shows an accelerated O_2-uptake per gram (fresh weight) after infection and incubation at 25° C. As the water content of all samples was the same, this respiration-gradient can not, either partly or wholly, be due to a decrease of the water content of the tissue near the area invaded by the fungus, which at calculating the O_2-uptake per gram of tissue would result in a respiration-gradient independent of the occurrence of a real increase of the respiration rate.

In the infected tuber halves an ever increasing respiration rate was usually found with decreasing distance to the surface. However, in some tubers the respiratory increase of the tissue adjacent to the zone with mycelium appeared to deminish again and sometimes the O_2-uptake dropped to a level lower than the normal respiration rate. This phenomenon was accompanied by a loss of turgescence in the

cells of the area adjacent to the infected parts. The development of such a zone with soft tissue in some tubers may be accompanied by a higher intensity of the fungal attack and a greater susceptibility to infection.

It was difficult to ascertain the exact relation between the extent of the respiratory increase and the intensity of infection, as a reliable standard of comparison has not yet been found. In fact the density of the aerial mycelium on the infection surface in relation to the depth over which the potato tissue had collapsed, could be observed at the end of the incubation, but these two phenomena could only be roughly estimated. When this was done, the results of many experiments suggested that in tuber halves without a zone with soft tissue the differences in respiratory increase as well as the distance from the surface where a respiratory increase could still be demonstrated, corresponded with the differences in intensity of infection and with the length of the period of incubation at 25° C. This is illustrated in Fig. 2 and 3.

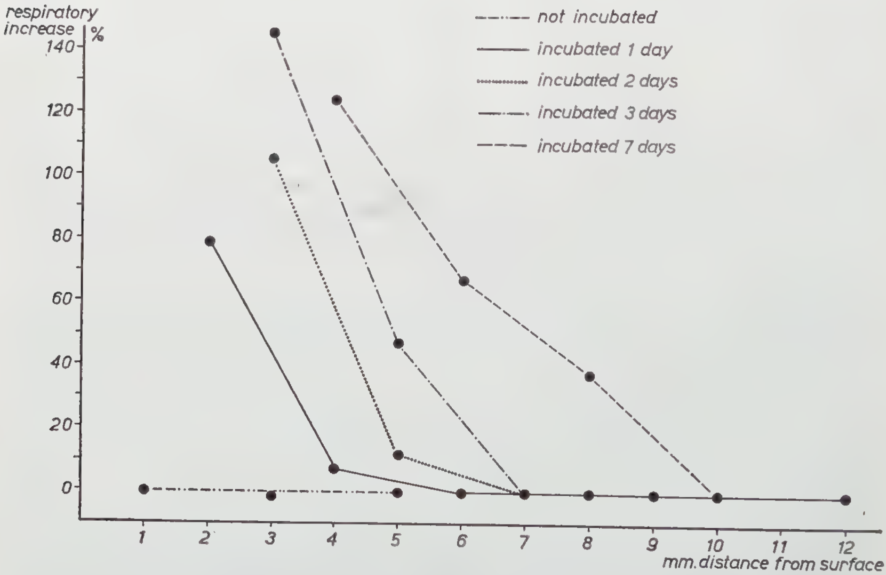

Fig. 2. Relation between respiratory increase after infection and the length of the period of incubation at 25° C.

It may be concluded that after infection of a tuber half with *Gibberella saubinetii* (*Mont.*) *Sacc.* the respiration of host tissue which was not invaded by mycelium, is accelerated. The extent of the acceleration is dependent on the distance to the area invaded by the fungus and on whether or not a zone with soft tissue had developed.

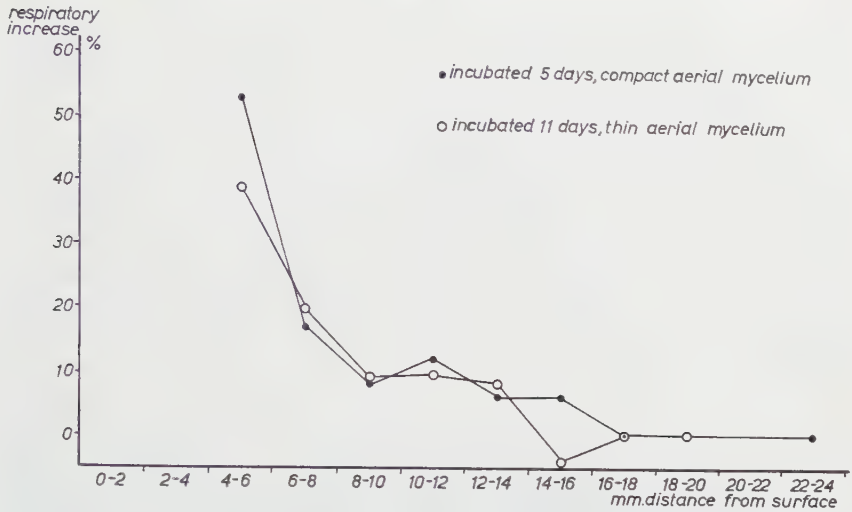

Fig. 3. Relation between respiratory increase after infection and the density of the aerial mycelium as well as the period of incubation at 25° C.

CHAPTER III

COMPARISON OF THE PATHWAYS FOLLOWED BY AEROBIC RESPIRATION IN HEALTHY AND IN DISEASED TISSUE

1. Introduction

A respiratory increase may be caused by an acceleration of the normal processes of which the respiration chain consists, by the development of pathways that under normal conditions are not present or are but slightly used, or by a combination of these possibilities. This aspect of the host-parasite relation has been scarcely investigated. Many investigations on the respiration after infection are not concerned with the respiratory pathway. Allen (1953) discusses a number of factors which might be rate-limiting in respiration and which might be changed under the influence of toxins excreted by the parasite, but this are factors which would allow the respiration to increase without altering the pathway.

Farkas and Kiraly (1955) and Kiraly and Farkas (1955) think that the respiratory mechanism of wheat leaf disks infected with *Puccinia graminis* is changed between the glycolysis and the terminal oxidation, as malonate much stronger inhibited the O_2-uptake of healthy tissue (60–70 %) than of infected disks (30–40 %). By iodoacetate and fluoride both tissues were equally affected. The latter observation was confirmed for other varieties of wheat infected with *Puccinia graminis* by Shaw and Samborski (1957), who studied in addition the rôle of the pentosephosphate cycle (hexosemonophosphate shunt, direct oxidative pathway; Horecker 1953) in respiration.

In the Embden-Meyerhof-Parnas pathway C_6 and C_1 from glucose are equally converted to CO_2 (C_6/C_1 ratio 1), but in the pentosephosphate cycle the C_6/C_1 ratio is <1, as in the beginning C_1 is more rapidly liberated in the form of CO_2 than C_6 is (Beevers and Gibbs 1954). In experiments with added glucose–1–C^{14} or glucose–6–C^{14} the C_6/C_1 ratio in the respiratory CO_2 of infected leaf parts appeared to be lower than that of healthy parts, indicating an increasing importance of the cycle in the respiration after infection. However, the cycle is reputedly resistant to the action of fluoride and iodoacetate. The % inhibition of the respiration of infected tissue caused by these compounds should have been lower than it is in healthy tissue and not equal, as was observed. Authors do not give a satisfactory explanation of these data.

A lower C_6/C_1 quotient in hypocotyls of safflower (*Carthamus tinctorius L.*) infected with *Puccinia carthami Cda.* especially in tissue containing sporulating pathogen (pycnospores, uredospores) might suggest an increase of the oxidation via the hexosemonophosphate shunt. This is in agreement with the decrease of the NaF-sensitiveness of the respiration (Daly and Sayre 1957; Daly, Sayre and Pazur 1957).

All three investigations mentioned above were carried out with the host-parasite complex. It is not known to what extent the metabolism of the parasite is responsible for the change in the respiratory pathway of the complex. However, according to Shu and Ledingham (1956) the hexosemonophosphate shunt plays an important part in the respiration of uredospores of wheat-stem rust. The supposed changes in the respiratory mechanism of safflower, which were especially noticeable with sporulating pathogen, probably should be attributed to an increase of the metabolism of the parasite.

In this chapter an attempt will be made to obtain evidence which may throw light on the question whether or not the respiratory mechanism is qualitatively changed in potato slices obtained from areas adjacent to the cells invaded by *Gibberella*.

2. Respiratory quotient

The respiratory quotient is not only dependent on the substrate used up, but is also determined by the products formed from it (Tamiya 1932). Although R.Q.-values are of secondary importance only, they may indicate changes in the metabolism appearing after infection.

The respiratory quotient was estimated by means of the direct method of Warburg. The gas exchange of each sample was measured first in the absence of alkali during two intervals of one hour; then 0.2 ml. KOH 10 % was added to the centre well and after 15 min. equilibration the O_2-uptake was measured for some hours. The R.Q.-values were calculated from the readings of the last hour before and the first hour after addition of KOH to the flask; in this manner the influence of the gradual increase in respiration rate during the experimental period remained small.

In table 3 the values are given for three experiments with both non-infected halves of some tubers which have been stored at 25° C. for some days after cutting. In general the R.Q. was <1.0 and showed a considerable variation. This agrees with other investigations (Boswell and Whiting 1938, Hellinga 1942). The variation is chiefly to be attributed to individual differences between the tubers. Usually the differences within a tuber half were small. The halves of a tuber gave nearly equal average values of the R.Q.

As appears from table 4, it was found for samples from the infected halves of potato tubers that the R.Q. was not affected in spite of the accelerated respiration of the tissue adjacent to the area invaded by the fungus.

It may be concluded that the R.Q. of the host cells which were not penetrated by the mycelium, was not changed.

TABLE 3. Respiratory quotients of samples from the two non-infected halves of three tubers. Tuber halves stored at 25° C. for resp. 10, 13 and 18 days.

storage at 25° C.	10 days		13 days		18 days	
tuber halves / mm. from the surface	I	II	I	II	I	II
5–7	0.79	0.85	0.98	0.98	0.80	0.87
7–9	0.84	0.84	0.93	1.01	0.82	0.84
9–11	0.92	0.81	0.97	1.05	0.80	0.84
11–13	0.91	0.81	—	1.00	—	0.85
13–15	0.85	0.84	—	1.00	—	0.80
15–17	—	—	—	0.95	—	0.82
average	0.86	0.83	0.96	1.00	0.81	0.84

TABLE 4. R.Q. and μl. O_2-uptake/hour/gram (fresh weight) of samples from the non-infected and the infected halves of two tubers, incubated after infection at 25° C. Infected halves without soft tissue.

mm. from the surface		incubated 15 days		incubated 22 days	
		R.Q.	μl. O_2	R.Q.	μl. O_2
non-infected half	5–7	0.96	85.8	0.95	98.8
	7–9	0.95	85.6	0.96	98.5
	9–11	0.95	84.7	0.99	99.0
	11–13	0.92	88.5	0.98	101.5
infected half	5–7	0.91	126.4	0.88	131.8
	7–9	0.91	107.3	0.90	102.8
	9–11	0.94	108.2	0.89	94.7
	11–13	0.93	94.8	0.92	90.8
	13–15	0.96	90.6	0.90	91.6
	15–17	0.96	90.2	0.90	92.4
	17–19	0.85	86.7	0.90	85.4

3. Experiments with respiratory inhibitors

The mechanism of respiration and its changes may be studied by the determination of the effect on the O_2-uptake of an addition of compounds which are inhibitory to one or more reactions of the respiratory chain. The value of conclusions from such experiments is highly dependent on the specificity of the inhibitory action. From the extensive review by James (1953) on the use of respiratory inhibitors, it is clear that no inhibitors are known that specifically interfere with one enzyme reaction, though the specificity might be enhanced by a careful choice of the dosage and the experimental conditions.

In experiments with intact cells it is necessary that the compounds should be taken up well. The great influence exercised by the pH of the medium on the inhibitory action of certain compounds, which by some authors is attributed to the effect of the pH on the entrance of the molecules into the cells (James 1953), made it necessary to replace distilled water as a medium for the potato slices by a buffer solution. For this a 1/15 M. phosphate buffer was selected (mixture of 1/15 M. KH_2PO_4 and 1/15 M. Na_2HPO_4 in water). In this buffer the O_2-uptake of the disks is only slightly enhanced. Within certain limits the pH of the medium appeared to have little effect on the respiration rate. In accordance with Boswell and Whiting (1938) and with Hellinga (1942) it was found that the O_2-consumption was the same at pH 5.5–6.8. Table 5 shows that this O_2-uptake (pH 6.2) was somewhat greater than in distilled water, and scarcely differed from that at pH 7.7 and pH 4.2.

Unless otherwise stated, a buffer pH 6.2 was used in experiments with added iodoacetate, sodium fluoride or sodium azide. When malonate was added, the disks were submersed in a buffer pH 4.3.

TABLE 5. μl. O_2-uptake/hour in distilled water or 1/15 M. phosphate buffer of samples of 10 disks from an untreated potato tuber.

medium	μl. O_2-uptake			
aqua dest.	16.5	16.0		
buffer pH 4.2	16.6	16.2	15.7	16.5
buffer pH 6.2	18.9	19.1	18.1	18.5
buffer pH 7.7	19.4	18.2	15.2	

In the experiments the additions of the inhibitor solved in water and adjusted to the proper pH, were made from the side-arms of the Warburg flasks after measurement of the respiration rate during the two preceding hours. After that the course of the O_2-uptake was watched for some hours. To the control samples corresponding amounts of distilled water were added.

In calculating the effect of the inhibitors the gradual increase of the respiration rate during the experimental period had to be taken into account. Therefore, in all experiments from each tissue zone a control sample was prepared besides the disks to which the compound was added. The same procedure was followed for the determination of the stimulatory effect of 2,4–dinitrophenol (Chapter v). For each

period of one hour after addition of the compound, the effect was calculated in % of the average respiration rate during the two hours before addition, and corrected for the increase of the respiration rate in the course of the experiment.

$$\% = 100 - \frac{\mu l.\ O_2 \text{ after addition}}{\mu l.\ O_2 \text{ before addition}} \times \frac{\mu l.\ O_2 \text{ control before addition}}{\mu l.\ O_2 \text{ control}} \times 100.$$

An inhibition is indicated by a positive %, a stimulation by a negative %.

a. *Experiments with mono-iodoacetate*

Iodoacetate is generally considered to be an inhibitor of glycolysis. This action is thought to be due to an irreversible reaction with –SH groups of enzyme protein. Although several enzymes contain these groups, and an inhibitory action on the activity of a number of these enzymes is known, *in vivo* the 3–phosphoglyceraldehyde (triose-phosphate) dehydrogenase would be affected preferentially, and the reaction in low concentrations of the inhibitor would be reasonably specific (JAMES 1953).

The experiments were carried out with potato-tissue slices in 1/15 M. phosphate buffer pH 6.2 to which iodoacetate was added, so that a concentration of 0.002 M. was obtained. The O_2-uptake was considerably reduced to a nearly constant, low level, which was reached 4–5 hours after addition of the inhibitor. In table 6 the data of 4 experiments are reviewed. It is clear that the % inhibition of the O_2-uptake of the samples from the infected tuber halves of experiments 2 and 4 were nearly the same, whether the respiration rate was increased by the disease or not, and were in close agreement with the inhibition in disks from non-infected tuber halves. The O_2-absorption was suppressed with 90–95 %. However, in the infected halves of the experiments 1 and 3 a zone with soft tissue had developed. The respiration rate of samples from this area (expt. 1:I, II and expt. 3:I) decreased when the samples were approaching the cells invaded by the fungus, and this decrease was accompanied by a

TABLE 6. $\mu l.\ O_2$-uptake/hour/gram (fresh weight) before addition of iodoacetate to 0.002 M. pH 6.2 and % inhibition 4–5 hrs. after addition. Samples cut from zones of non-infected and infected halves of 4 tubers. Zones I nearest the surface at > 3 mm. distance.

samples		expt. 1		expt. 2		expt. 3		expt. 4	
		$\mu l.\ O_2$	% inhibition	$\mu l.\ O_2$	% inhibition	$\mu l.\ O_2$	% inhibition	$\mu l.\ O_2$	% inhibition
non-infected	I	54.6	94	50.7	96	54.4	95	53.6	99
	II	53.8	94	49.1	95	54.9	95	46.1	98
infected	I	*40.1*	*66*	111.1	91	*55.2*	*78*	88.5	92
	II	*53.5*	*85*	87.4	92	69.4	92	79.1	91
	III	56.7	89	75.0	93	55.0	93	70.1	93
	IV	53.2	92	68.1	94	42.7	93	66.6	86
	V	44.4	95	67.8	94	50.4	92	62.2	94
	VI	35.9	96	53.5	95	51.8	92	—	—

decreasing inhibition by iodoacetate. The % inhibition in the other samples had not changed.

b. *Experiments with sodium fluoride*

Sodium fluoride has an inhibitory effect on several enzymes, but enolase, which catalyses a reaction of the glycolysis chain, would be the most sensitive of them and this enzyme would be nearly specifically inhibited in low concentrations of the inhibitor. The inactivation of enolase is dependent on the presence of inorganic phosphate and magnesium (Sumner and Myrbäck 1951, Miller 1958).

In the experiments sodium fluoride was added to potato slices submersed in 1/15 M. phosphate buffer pH 6.2, so that a concentration of 0.02 M. was obtained. The development of full inhibition required 4–5 hours, after which the O_2-uptake was suppressed with about 80 %. Just as in the experiments with iodoacetate the % inhibition after addition of NaF were the same in samples from the non-infected and the infected tuber halves, except in those samples that has been cut from the area with soft tissue that occurred in some infected tuber halves (table 7). The O_2-uptake of these disks was inhibited to a lower degree.

TABLE 7. μl. O_2-uptake/hour/gram (fresh weight) before addition of sodium fluoride to 0.02 M. pH 6.2 and % inhibition 4–5 hrs. after addition. Samples cut from zones of the non-infected and the infected halves of a tuber. Zones I nearest the surface at 5 mm. distance.

tuber halves	non-infected		infected					
samples	I	II	I	II	III	IV	V	VI
μl. O_2-uptake	53.9	53.5	*47.9*	56.3	51.4	45.0	43.7	39.9
% inhibition	80	82	*68*	80	78	80	81	79

c. *Experiments with sodium azide*

According to James (1953) sodium azide influences the metabolism by the formation of metal complexes, by which cytochrome oxidase may be markedly inhibited. Polyphenol oxidase is reported to be partially inhibited *in vitro*. Moreover, sodium azide interferes with the phosphate transfer and the formation of high-energy phosphate. Both actions are reversible after removal of the azide. The degree of inhibition of the metal-containing enzymes is at pH 5–8 highly dependent on the pH of the experimental medium. Now the latter phenomenon was studied with potato tissue too.

As shown in table 8, the inhibition of the O_2-uptake of potato disks was much greater at a lower pH. As in all experiments with azide the inhibition rapidly developed and the respiration rate reached a nearly constant level already 2–3 hours after addition of the inhibitor, it seems reasonable to suppose that the differences in inhibition are a result of the effect exercised by the pH on the metal-complex formation rather than of its effect on the entrance of the azide into the cells. In conclusion, the inhibition of the terminal oxidase(s) was probably dominant.

The experiments with disks from non-infected and infected tuber halves were carried out in phosphate buffer pH 6.2 to which sodium azide was added, so that a concentration of 0.002 M. was obtained.

TABLE 8. Inhibition of the O_2-uptake of healthy potato-tuber tissue after addition of sodium azide to 0.002 M. to media (1/15 M. phosphate buffer) with pH 7.7, 6.2 or 4.2.

pH medium	% inhibition					
7.7	50	59	53	54	56	
6.2	83	81	84	84	88	89
4.2	94	95				

From the experiment of table 9 it is clear that a stimulation of the respiration occurred in the samples of the infected half near the area invaded by the fungus, but that the % inhibition were nearly the same in all samples and in both halves.

Data on the effect exercised by sodium azide on the respiration of samples from tuber halves with a zone with soft tissue are not available.

TABLE 9. μl. O_2-uptake/hour/gram (fresh weight) before addition of sodium azide to 0.002 M. pH 6.2 and % inhibition 3 hrs. after addition. Samples cut from zones of the non-infected and the infected halves of a tuber. Zone I nearest the surface at 6 mm. distance.

tuber halves	non-infected		infected					
samples	I	II	I	II	III	IV	V	VI
μl. O_2-uptake	53.7	53.4	75.9	70.5	59.5	50.0	48.3	54.8
% inhibition	86	89	87	87	91	91	89	86

d. *Experiments with malonate*

In the argumentation for the participation of the tricarboxylic acid cycle in the respiration of a tissue the inhibition of the O_2-uptake by malonate plays an important part. However, malonate is not a specific inhibitor, although it is generally assumed that it primarily gives a competitive inhibition of succinic dehydrogenase with *in vitro* an affinity ratio malonate/succinate of about 50/1 in favour of the inhibitor (JAMES 1953). HANLY, ROWAN and TURNER (1952), discussing the literature, conclude that it seems quite likely, at least in experiments with higher concentrations of malonate and especially where succinate accumulation can not be demonstrated, that its effect on respiration can not safely be ascribed to an effect on succinic dehydrogenase.

Whether the tricarboxylic acid cycle is active in the respiration of potato-tuber tissue is not certainly known. Succinic dehydrogenase is reported to occur in the potato (MILLERD 1951, HACKETT 1956). The inhibition by malonate of the O_2-absorption of potato slices, as discussed below, would be in agreement with this view. However, evidence has been obtained for a not entirely specific inhibitory action of malonate, at least when the inhibition is greater than about 25 %.

In the experiments a solution of malonic acid, the pH of which was

adjusted with NaOH, was added to potato-tuber slices submersed in 1/15 M. phosphate buffer with the same pH. In accordance with the results obtained with carrot disks by HANLY, ROWAN and TURNER (1952), the degree of inhibition depended on the concentration of the inhibitor and on the pH of the medium, a lower pH promoting the inhibition. At pH 6.5 the O_2-uptake of healthy tissue was inhibited by 0.1 M. and 0.17 M. malonate with about 25 % and 40 % respectively. At pH 4.3 0.01 M. malonate caused an inhibition of about 30 % already, whereas the O_2-consumption was suppressed with about 80 % by 0.06 M. malonate.

If the tricarboxylic acid cycle is active in potato-tuber tissue and if malonate inhibits the O_2-uptake exclusively by a competitive action on the succinic dehydrogenase, one may expect to observe a reversal of the inhibition as a result of the accumulation of succinic acid in the cells owing to this inhibition or after addition of this acid to the medium. However, table 10 shows that a self-recovery of the O_2-uptake was only observed when the respiration was but slightly inhibited.

From table 11 it appears that in agreement with these results a greater inhibition by malonate of the O_2-uptake was not reversed after addition of succinate. Nevertheless the development of the

TABLE 10. % Inhibition of the O_2-uptake of healthy potato-tuber tissue by malonate in buffer pH 6.5 for each hour after addition of the inhibitor. Self-recovery in 0.1 M. malonate pH 6.5.

hrs. after addition	1st	2nd	3rd	4th	5th	6th
0.1 M. malonate	—11 %	27 %	18 %	10 %	0 %	—
	— 4 %	20 %	14 %	10 %	6 %	—
0.17 M. malonate	— 8 %	27 %	33 %	33 %	40 %	40 %
	—16 %	22 %	23 %	37 %	35 %	41 %

TABLE 11. Effect of succinate or fumarate on % inhibition of the O_2-uptake of healthy potato-tuber tissue by malonate added simultaneously or 4 hrs. before. Medium: 1/15 M. phosphate buffer pH 4.3.

hrs. after addition	1st	2nd	3rd	4th	5th	6th	7th
0.04 M. malonate	11 %	53 %	64 %	64 %	82 %	72 %	76 %
	16 %	51 %	61 %	66 %	79 %	69 %	73 %
0.04 M. malonate 0.05 M. succinate	—19 %	3 %	42 %	47 %	61 %	68 %	73 %
					+ 0.05 M. succinate		
0.04 M. malonate	18 %	54 %	65 %	71 %	77 %	75 %	73 %
	11 %	51 %	64 %	72 %	73 %	73 %	70 %
0.03 M. malonate 0.012 M. fumarate	2 %	17 %	21 %	37 %	55 %	64 %	75 %
					+ 0.012 M. fumarate		
0.03 M. malonate	8 %	42 %	62 %	71 %	78 %	74 %	70 %

inhibition was distinctly retarded by simultaneous addition of succinate and malonate, yet after a few hours the degree of inhibition was nearly equal to that obtained with the same concentration of malonate without succinate. Similar results were obtained with fumarate.

That in these experiments succinate had no effect on the ultimate inhibitory effect exercised by malonate on the O_2-absorption might be explained from the great affinity of the inhibitor to the succinic dehydrogenase. However, the experiments with fumarate as well as the absence of a self-recovery when the inhibition was greater than 25 %, suggest that the results are to be understood as an indication that malonate did not attack succinic dehydrogenase exclusively.

Experiments with disks from the non-infected and the infected halves of potato tubers were performed with 0.06 M. malonate pH 4.3. The respiration rates and the inhibition by malonate are represented for two experiments in table 12. Clearly the inhibition of the O_2-uptake of the samples from the infected halves was not the same. A stimulation of the respiration after infection was not immediately accompanied by a change in the degree of inhibition by malonate. Only when the influence of the infection continued, and the respiration rate was accelerated to a greater extent, the % inhibition distinctly decreased.

TABLE 12. μl. O_2-uptake/hour/gram (fresh weight) before addition of malonate to 0.06 M. pH 4.3, and % inhibition after addition. Samples cut from the zones of non-infected and infected halves of 2 tubers. Medium: 1/15 M. phosphate buffer pH 4.3.

tuber halves	mm. distance from surface	expt. 1		expt. 2	
		μl. O_2	% inhibition	μl. O_2	% inhibition
non-infected	5–7	43.0	77	39.6	72
	7–9	45.4	79	38.8	76
infected	5–7	81.8	21	118.8	38
	7–9	66.7	52	83.1	52
	9–11	57.6	67	66.9	74
	11–13	34.8	66	54.6	73
	13–15	37.3	67	52.1	75

The O_2-consumption of the two samples from the non-infected halves was equally retarded. The differences in the inhibition of the normal respiration of tissue from both halves of these tubers did not exceed the differences in % inhibition observed in preliminary experiments with disks from two non-infected halves of a tuber.

Data concerning the inhibition by malonate in samples from infected tuber halves containing a zone with soft tissue are not available.

4. Discussion

From the experiments with inhibitors described above no definite conclusions can be drawn concerning the pathway of aerobic respiration, as it is not known, whether the compounds have affected enzymes other than those that would have been attacked preferentially. The data obtained after addition of malonate suggest that the inhibitory action of this compound was not limited to the succinic dehydrogenase.

Concerning the question whether a qualitative change occurs in the respiratory pathway in consequence of the disease, it is clear that in tissue from the infected tuber halves the % inhibition after addition

of iodoacetate, sodium fluoride or sodium azide were the same for all samples, whether or not the respiration rate was increased after infection, provided that the samples were cut from apparently normal tissue. In conclusion, these experiments provided no evidence of a qualitative change of the respiratory pathway.

On the other hand, the inhibition by malonate did not prove to be the same for all samples. Although stimulation of the respiration after infection was not immediately accompanied by a change in the % inhibition, yet when the influence of the infection continued and the O_2-uptake was increased to a greater extent, the % inhibition considerably decreased. This indicates that with continued influence of the infection the respiratory pathway was qualitatively changed too.

In the zone with soft tissue which developed in some tubers after infection the tissue-structure was affected and the cells had lost their turgescence. The desintegration of tissue not penetrated by mycelium was accompanied by a decrease of the inhibition by iodoacetate and sodium fluoride, whereas the respiration rate was increased to a smaller extent than in the adjacent normal cells. So, within this area changes in the respiratory pathway had developed which were not observed outside this area. Maybe these changes are connected with the dying of the cells.

Of the apparently normal tissue it may be said that at least during the initial stages of respiratory stimulation after infection no evidence of a qualitative change of the pathway was obtained, whereas after addition of iodoacetate, sodium fluoride or sodium azide no evidence at all was found in support of such a change. The unaltered R.Q. after respiratory increase was in agreement with these facts.

CHAPTER IV

CHANGES IN CARBOHYDRATE CONTENT AND THEIR RELATION TO THE PARASITICALLY INCREASED RESPIRATION

1. Introduction

In the preceding experiments with infected potato tubers evidence for a qualitative change of the respiratory pathway was only obtained after addition of malonate, and then only in advanced stages of respiratory stimulation. The onset of the stimulation is likely to be merely quantitative, and might be attributed to an increase of the intensity or/and the capacity of the processes which regulate the respiration rate.

It is generally assumed that in potatoes carbohydrates are the ultimate respiratory substrate. Hopkins (1927) found that after wounding potato tubers the content of total sugars or reducing sugars was altered parallel with the respiration rate. Appleman and Miller (1926) studied tubers in various stages of development, and Barker (1935/36) treated mature tubers with low temperatures. These authors observed a similar behaviour of the respiration intensity and the sucrose

content. In infected tuber halves starch disappeared from the tissue adjacent to the area invaded by *Gibberella* (Richter, unpublished) and it is clear from the experiments described in Chapter II that in this tissue the O_2-uptake is stimulated.

In this chapter experiments will be described bearing on the question whether there exists a close relation between the O_2-consumption and the sugar content, and whether the respiration rate is regulated by the amount of substrate. As the respiration intensity may be related with one special sugar, it is necessary to determine not only the amount of total sugars or reducing sugars in relation to the O_2-uptake, but, if possible, also to determine the content of the various sugars separately.

Therefore, after measurement of the O_2-absorption, extracts were prepared from disks out of the non-infected and the infected tuber halves, and the amounts of the respective sugars were determined after quantitative separation using paper-chromatography.

2. Methods

a. *Colorimetric estimation of sugars*

Dissolved carbohydrates were estimated in 1 ml. samples using the colorimetric method with anthrone reagent according to Mokrasch (1954). In view of the rapidity of colour formation with the respective sugars, the mixture of the reagent with fructose was heated to 80° C. ± 0.5° C. for exactly 5 min. and the mixture with glucose or sucrose for exactly 25 min.

The colour densities were measured with a Gallenkamp colorimeter (King 1951) provided with a heat-filter. A colour-filter (Ilford nr. 607) transmitted from the entering light mainly the light with a wave length of 620 mμ. The lightpath through the solution in the absorption cells was 1 cm.

From estimations with watery solutions of sucrose, glucose or fructose with a concentration of up to resp. 80 μg./ml., 40 μg./ml. and 40 μg./ml. it appeared that after reaction with anthrone the extinctions were proportional to the amount of sugar. It proved to be possible to carry out the estimations correct to 1–2 μg.

b. *The preparation of extracts from potato-tuber tissue*

The tissue disks were killed rapidly in boiling ethanol 80 % to which was added a little $CaCO_3$ and a few drops of a satured solution of $CuSO_4$ in water, and cooled after about 10 min. boiling. The disks were extracted further in a Soxhlet apparatus containing 80 % ethanol.

Preliminary experiments showed that the extraction of 100 and 500 disks was complete after 3–4 hours. Therefore after 3–4 hours extraction the solution was removed from the Soxhlet into an Erlenmeyer flask, the ethanol was distilled off, and the remaining watery extract was concentrated to about 2 ml. by slightly heating in vacuo. After transference of the extract to a little flask, and rinsing the flask in which it was concentrated, the end-volume was about 3 ml. These extracts could be used in paper-chromatography without further treatment.

TABLE 13. Recovery of sucrose, glucose and fructose from 20 ml. of a solution of each sugar in water concentrated to about 3 ml. and estimated in 0.1 ml. (fructose 0.2 ml.) diluted with water to 1 ml. Values in μg./0.1 ml. (fructose 0.2 ml.) of the concentrated solution.

μg. sucrose		μg. glucose		μg. fructose	
calculated	recovered	calculated	recovered	calculated	recovered
46	46	44	42	44	43
46	46	44	44	44	43

As appears from table 13, it could be ascertained in experiments with known sugar solutions that no sugar was lost in concentrating 20 ml. to about 3 ml. provided that the flask in which it was concentrated, was rinsed with warm, distilled water after transference of the concentrated solution to a small flask. The differences observed correspond with the normal variation of estimations using anthrone.

c. *Paper-chromatography*

Chromatograms were prepared on water-washed Whatman nr. 1 paper and run with propanol-ethylacetate-water (7:1:2; v/v/v) as a solvent in closed glass jars placed in a room with a constant temperature of 21° C. The descendent technique was used. On the starting-line 0.25 ml. extract was applied in a narrow strip 2 cm. in length (Fig. 4) using a micro-pipette (AGLA Micrometer Syringe, Burroughs Wellcome & Co., London). Before applying extract again on the same place, the paper was dried with warm air (40°–50° C.). On both sides at some distance from the extract a mixture of sucrose, glucose and fructose was applied. A complete separation of the sugars required about 40 hours. The solvent front ran from the teeth cut in the end of the paper.

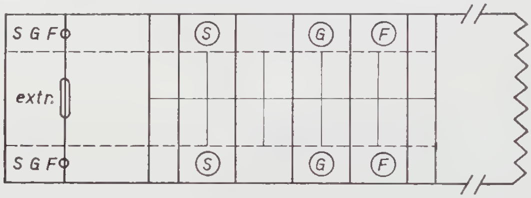

Fig. 4. Scheme of a chromatogram.
S = sucrose G = glucose F = fructose

To determine the running-speed of some sugars and to identify the sugars that occur in potato extract, chromatograms with extract and known sugars were run with propanol-ethylacetate-water (7:1:2; v/v/v) or n-butanol-ethanol-water (9:1:10; v/v/v) for 40 and 60 hours respectively. After drying the paper was sprayed with an aniline phthalate-, a naphthoresorcinol- or a benzidine-reagent (Cramer 1953, Linskens 1955). The coloured spots obtained with extracts appeared indistinguishable from those formed by known sucrose, glucose and fructose. In the concentrations of extract used, never more than these three spots were detected, which is in agreement with the observations of Le Tourneau (1956). It is assumed, that the sugars detected in the extracts were sucrose, glucose and fructose.

d. *Quantitative estimation of sugars from paper-chromatograms*

With water the sugars could be easily eluted from the chromatogram papers. However, as Whatman nr. 1 paper appeared to contain water-soluble compounds which produced a colour with the anthrone reagent, the paper was thoroughly washed with distilled water and dried before use. After this the interference was slight and was corrected by comparison with blank estimations.

The sugars of the extracts were localized by means of the mixture of sucrose, glucose and fructose chromatographed on the same paper. Blank estimations were carried out with pieces of paper cut from the parts of the chromatogram just before the area with sucrose and just after that with fructose (Fig. 4). The strips containing sugars and the strip between those with sucrose and with glucose were cut out and divided into 4 equal parts. All pieces were eluted separately with distilled water to obtain 1 ml. eluate. This treatment had to be carried out with extreme care, as paper fibrils after hydrolysis in the anthrone reagent would react as sugars.

Some estimations were carried out with chromatograms on which mixtures of known amounts of sucrose, glucose and fructose had been separated. The amounts which were applied and recovered, are given in table 14. It appears that the determination after chromatographic separation was mostly accompanied by some loss of sugar, but the differences did not exceed 10–15 %.

In table 15 are represented the results of estimations in two extracts from 250 potato slices, all cut out of one tuber. To one extract known amounts of sucrose, glucose and fructose were added, after which both extracts were concentrated to 3.4 ml. From chromatograms with 0.01 ml. extract the amounts of sugars were determined and calculated in μg. per disk.

Assuming that the content of the various sugars originally was the same in both extracts, it may be concluded that the % sugar which was recovered agreed with the % found with a solution of pure sugars.

From the values of table 15 it can be calculated that 10 disks contain sufficient extractable sugars for an estimation using paper-chromatography, if 0.25 ml. of the extracts concentrated to about 3 ml. was used.

TABLE 14. Recovery of sucrose, glucose and fructose from chromatograms of mixtures of these sugars.

sugars	μg. applied	μg. recovered			% recovery
sucrose	70	70	63	64	90–100
	35	32	38	33	90–110
glucose	40	38	37	41	90–105
	20	17	20	17	85–100
fructose	40	41	36	37	90–105
	20	18	19	20	90–100

TABLE 15. Sucrose, glucose and fructose in μg./disk of potato-tuber tissue estimated in two parallel extracts of 250 disks, concentrated to 3.4 ml. To one extract known amounts of the sugars were added before concentration. Estimations from chromatograms of 0.01 ml.

sugars	extract I	extract II + sugars	μg. recovered	μg. added	% recovery
sucrose	45 μg.	69 μg.	24	27	89
glucose	51 μg.	66 μg.	15	16	94
fructose	38 μg.	52 μg.	14	16	88

3. Results and discussion

Experiments were carried out in spring (April–June) and in autumn (September). In all tubers a clear respiratory increase was observed after infection. The amounts of sucrose, glucose and fructose were determined in duplo, and the average values calculated in μg. per 0.1 gram of tissue were plotted against the O_2-consumption in μl. per 0.1 gram.

The results of two experiments in spring are drawn in Fig. 5. Although in each experiment, as a result of the relatively narrow area with stimulated respiration after infection, few observations were obtained of samples with different respiration rates, it is clear that the fructose content is not altered parallel with the respiration rate. Experiment 1 shows a more or less parallel behaviour of the O_2-consumption and the amounts of sucrose and glucose, but from experiment 2 it appears that this relation is absent.

If it is assumed that the observations obtained with samples from different tubers may be taken together, and for all experiments carried out in spring (5 expts. including those of Fig. 5) the average O_2-absorption is calculated of samples with a sucrose content of 120–140 μg.,

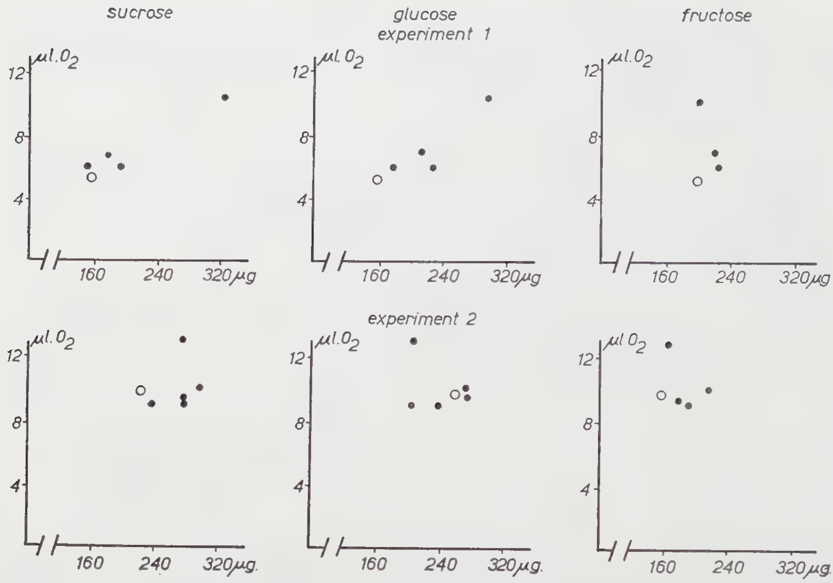

Fig. 5. μl. O_2-uptake and μg. sucrose, glucose and fructose per 0.1 gram (fresh weight). The sugar values are averages of duplicate determinations.
Experiments carried out in spring.
● values of samples from the infected halves.
○ average values of samples from the non-infected halves.

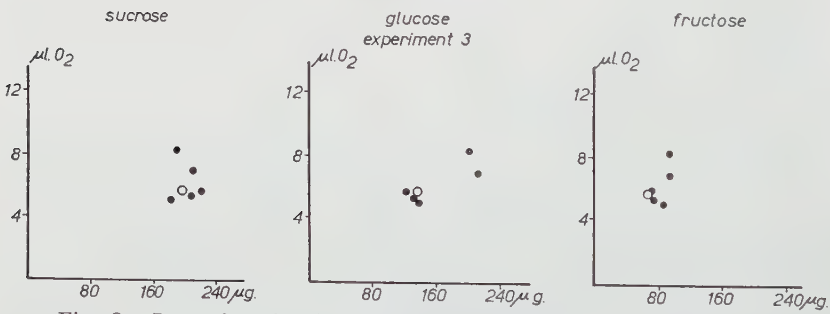

Fig. 6. Legends as in Fig. 5. Experiment carried out in September.

140–160 μg., 160–180 μg. etc. per gram of tissue, it appears from Fig. 7A that the relation between O_2-uptake and sucrose content might be understood as a relation between the rapidity of an enzyme reaction and the concentration of the substrate. This is not true for the relation between the O_2-uptake and the glucose content, when calculated and plotted in the same way (Fig. 7B).

However, from the experiment of Fig. 6 it is clear that in September an acceleration of the respiration after infection is not accompanied by an increasing sucrose content.

These conflicting results indicate that the extra O_2-consumption after infection is not due to an increase of the content of sucrose and glucose. Moreover, in the literature there is evidence that the respiration rate in the potato tuber is limited by other factors than the concentration of the substrate. HUELIN and BARKER (1939) found that the increase of the O_2-uptake of ethylene-treated potato tubers was accompanied by an increase of the sugar content, except when the experiments were carried out with tubers shortly after lifting. CALO and VARNER (1957) concluded from the influence of chloramphenicol on the acceleration of the O_2-absorption of potato-tuber disks during aeration that the protein synthesis is a necessary part of the mechanism by which the increased respiration develops. Moreover, the experiments with 2,4-dinitrophenol as described in Chapter v do not support the view that the respiration rate is regulated by the amount of available substrate.

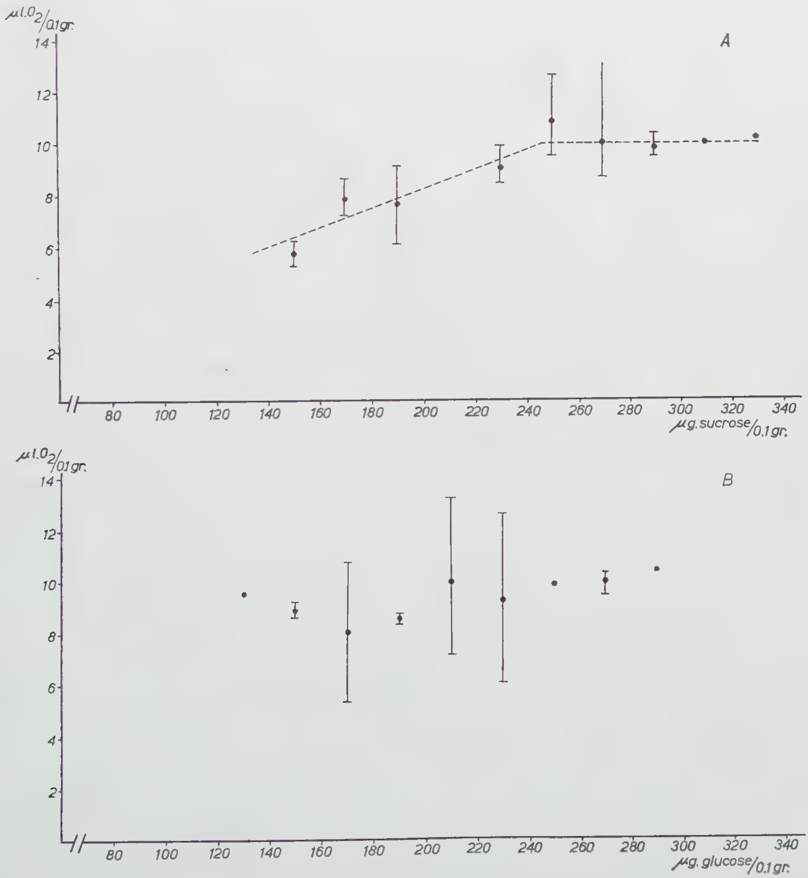

Fig. 7. Average μl. O_2-uptake/0.1 gram of the samples of all 5 experiments carried out in spring, calculated for the samples containing 120–140 μg., 140–160 μg. etc. sucrose (A) or glucose (B) per 0.1 gram (fresh weight).

On account of these data it is thought that the changes in the sugar content after infection are a parallel phenomenon resulting from the general activation of metabolism which includes the increase of the O_2-uptake, rather than the regulating mechanism of the respiration rate.

The importance of an increase in sugar content might be that a considerable stimulation of the respiration is made possible because in this case there is no danger that the substrate supply will become rate-limiting.

CHAPTER V

THE EFFECT OF 2,4-DINITROPHENOL ON THE OXYGEN CONSUMPTION

1. Introduction

The preceding experiments yield no evidence for a qualitative change of the respiratory pathway during the initial stages of the reaction to infection. Therefore, the most simple explanation of the increase of the O_2-uptake is an influence of the disease on the regulating mechanism of the respiration rate.

In preparations of cellular particles (mitochondria ?) from avocado fruits (Millerd, Bonner and Biale 1953), cauliflower (Laties 1953) and sweet potato (Akazawa and Uritani 1954) it appeared that there exists a coupling between the oxidative and the phosphorylating processes. After addition of adenylate to cauliflower preparations oxidizing succinate or α-ketoglutarate, the O_2-absorption increased (Laties 1953). This may indicate a regulation of the respiration rate by the oxidative phosphorylation, also in intact plant tissue. Then the respiration would proceed dependent on the presence of sufficient inorganic phosphate, which is bound to high-energy phosphate ($\sim$P), and on the regeneration of phosphate acceptors, the latter being determined in turn by the activity of transphosphorylating systems and the rate of utilization of phosphorylated acceptors in cellular reactions. If in potato-tuber tissue the respiration rate is limited by the phosphate-acceptor regeneration, it may be expected that influences which accelerate the regeneration and the $\sim$P-consumption, will increase the respiration rate.

Much of the evidence on the regulation of the respiration in plant tissue by a coupled phosphorylating-mechanism has been obtained from experiments with "uncoupling agents", especially with 2,4-dinitrophenol (DNP). According to Laties (1957) substrate level phosphorylation is shown to be resistent to DNP, and its action applies only to phosphorylation during electron transport. There is no unanimity of opinion whether DNP really uncouples the respiration from the phosphorylation (Loomis and Lipmann 1948, 1949; Bonner 1949), or whether this compound interferes in the metabolism after the first phosphorylation-reaction has taken place and $\sim$P-compounds may have been formed already (Teply 1949; Lardy and Wellman

1952, 1953; LATIES 1953; ELIASSON and MATTHIESEN 1956). BRONK and KIELLEY (1958) and Löw et al. (1958) studied the stimulation by DNP of the ATP [1])-ase action and the inhibition by DNP of the ATP-inorganic P^{32} and the ATP-ADP^{32} exchange reactions in preparations of rat-liver mitochondria. It is suggested that the stimulated ATP-ase action is the reversal of the phosphorylating reactions, and that DNP uncouples the phosphorylation by stimulating the break-down of a high-energy, non-phosphorus-containing intermediate in the enzyme complex. COHN (1953), COHN and DRYSDALE (1955) and DRYSDALE and COHN (1958) conclude from their experiments with the same object, using O^{18}-labelled inorganic phosphate or water, that all effects of DNP can not yet be explained by the assumption that there exists one place of action in the electron-transport system. There is evidence in favour of a DNP-action after the formation of a $\sim$P-bond, but an action before the formation of this bond could not be excluded.

Whatever the details of the mechanism of action of DNP may be, all authors agree that DNP abolishes or reduces the rate-limiting action of the phosphorylation. If the O_2-uptake of a tissue is stimulated after addition of DNP, this indicates that the respiration rate is limited either by the amount of endogenous phosphate acceptor and its regeneration rate or by the amount of inorganic phosphate. LATIES (1957) mentions a number of investigations with plant tissues in which after addition of DNP the respiration was distinctly increased a.o. with potato-tuber tissue (Sharpensteen 1953).

In this chapter experiments will be described concerning the effect of DNP-treatment on the O_2-uptake of potato-tuber disks after stimulation of the respiration due to infection.

2. METHODS

The effect of DNP on the O_2-uptake depends on the concentration of DNP in the medium and on the pH. Whereas DNP in certain concentrations stimulates the O_2-uptake, the latter is inhibited by higher concentrations. A lower pH of the medium intensifies the DNP-effect. In preliminary experiments with potato disks at pH 5.0 the greatest stimulation of the O_2-uptake was observed in 10^{-3} M. DNP, whereas 2.5×10^{-3} M. was inhibitory.

From each of a number of zones of the infected tuber halves 20 disks were cut and divided at random into two samples of 10 disks, one of which was used as a control by addition of distilled water instead of DNP. The respiration rate of the disks submersed in water was measured during two hours, and then DNP was added from the side-arms of the Warburg flasks to obtain a concentration of 10^{-3} M. After addition the course of the O_2-uptake was watched for some hours. The pH of the DNP-medium was 5.0.

As the O_2-uptake had reached its highest value in the second hour after addition of DNP, the DNP-effect was calculated in μl. O_2-

[1]) ATP = adenosinetriphosphate.
ADP = adenosinediphosphate.

uptake/hour/gram (fresh weight) and in % by comparison of the respiration rate during this hour with the average rate during the two hours before addition. Both values were corrected for the respiratory increase of the control samples during the same period as follows:

$$\text{DNP-effect } \mu\text{l. } O_2 = \text{increase } \mu\text{l. } O_2 \text{ in DNP} - \text{increase } \mu\text{l. } O_2 \text{ control}$$

$$\text{DNP-effect } \% = 100 \times \frac{\mu\text{l. } O_2 \text{ in DNP}}{\text{initial } O_2\text{-uptake}} \times \frac{\text{initial } O_2\text{-uptake control}}{\mu\text{l. } O_2 \text{ control}} - 100$$

3. Results and discussion

In experiments with healthy potato-tuber tissue the O_2-uptake was markedly stimulated by 10^{-3} M. DNP at pH 5.0, the R.Q. being 1.0–1.1, a fraction higher than in water. This reaction to the addition of DNP indicates that the respiration rate of healthy tissue is regulated by the coupled oxidative phosphorylation, which in its turn depends on the amount of phosphate acceptor and its rate of regeneration or on the inorganic phosphate content.

It is not likely that the amount of inorganic phosphate is the rate-limiting factor. As has already been stated in Chapter III (table 5) the O_2-uptake in 1/15 M. phosphate buffer was slightly larger than in water. This extra O_2-uptake is considered to be a salt respiration rather than a consequence of the increased concentration of inorganic phosphate in the cells. For the respiration is also accelerated in solutions of other salts (Steward, Stout and Preston 1940). In conclusion, the phosphorylation rate is most likely dependent on the transphosphorylating system and the regeneration of phosphate-acceptor sites.

In table 16 the results are represented of 4 experiments with tubers the two halves of which were placed at 25° C. during resp. 0, 3, 8 and 11 days after infecting one half of each tuber. For all zones the O_2-uptake of the control samples without addition of DNP is also given.

The samples of experiment I were cut immediately after infecting one of the two halves. As appears from column A, their O_2-uptake was equal. After incubation at 25° C. a distinct respiratory increase was found near the surface of the infected tuber halves of the expts. II–IV. In expt. IV this increase was lower in sample inf. 1 than in sample inf. 2 as a result of the occurrence of a zone with soft tissue. In column B the respiration rates during the second hour after addition of DNP or distilled water are presented. The O_2-absorption of all samples, except that of sample inf. 1 of expt. IV, appears to have been stimulated by DNP. In DNP-medium the O_2-uptake of the samples inf. 3–5 was practically equal within each tuber half, but the uptake of samples with a much increased respiration after infection reached higher values (expts. II and III inf. 1 and 2; expt. IV inf. 2).

The stimulation by 10^{-3} M. DNP was calculated in μl. O_2 (column C) and in % (column D). The O_2-uptake of the samples from the infected half of expt. I, which did not show a respiratory increase, was stimulated to the same extent. In the other experiments the stimulation by DNP decreased with the acceleration of the respiration after infection.

TABLE 16. The effect of 10^{-3} M. 2,4-dinitrophenol (DNP) at pH 5.0 on the O_2-uptake in μl./gram (fresh weight) in 4 experiments. Samples cut from the non-infected and the infected tuber halves, incubated at 25° C. for resp. 0–3–8–11 days. Samples inf. 1 nearest the surface. For all zones the O_2-uptake of the control samples without addition of DNP is given too.

			initial μl. O_2-uptake (A)				μl. O_2-uptake after addition of DNP or water (B)				Stimulation by DNP corrected for increase of the controls: μl. O_2 (C)				% (D)			
experiments			I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
non-infected tuber halves		+ DNP	46	66	52	54	127	138	135	125	60	52	69	55	89	59	101	74
		+ aq. dest.	*46*	*65*	*48*	*49*	*67*	*85*	*62*	*65*								
infected tuber halves	zones																	
	1	+ DNP	47	155	135	**96**	128	181	160	**99**	47	4	7	**0**	61	2	5	**0**
		+ aq. dest.	*49*	*147*	*136*	**85**	*83*	*169*	*154*	**88**								
	2	+ DNP	42	115	98	107	106	162	139	141	42	24	20	19	69	17	15	15
		+ aq. dest.	*45*	*113*	*89*	*107*	*67*	*136*	*110*	*122*								
	3	+ DNP	45	65	67	73	105	119	125	109	44	34	35	23	70	41	36	26
		+ aq. dest.	*43*	*68*	*62*	*70*	*59*	*88*	*85*	*83*								
	4	+ DNP	45	51	56	41	101	99	129	100	41	42	55	44	68	74	75	80
		+ aq. dest.	*44*	*54*	*57*	*42*	*59*	*60*	*75*	*57*								
	5	+ DNP	—	—	50	42	—	—	125	101	—	—	52	43	—	—	69	71
		+ aq. dest.	—	—	*48*	*40*	—	—	*71*	*56*								

It is conspicuous that the respiratory stimulation by DNP in the samples not affected by the infection (inf. 4 and 5) differs from that in tissue from the non-infected tuber halves. From preliminary experiments with some tubers the two non-infected halves of which had been stored at 25° C. for some days, it appeared that the respiration rate and the stimulation by DNP in the tissue from the two halves of a tuber showed similar differences. These differences are caused by the variation existing between the two halves of a tuber, and are not a result of the infection. Therefore, the respiration rates and their stimulation should be compared within each tuber half.

In all samples from the infected tuber halves, except in sample inf. 1 of expt. IV, a stimulation by DNP was observed. This suggests that the respiration rate in this tissue was limited by the phosphorylation, even after a large increase of the respiration due to infection. The DNP-effect calculated in % as well as the effect in μl. O_2 decreased after the acceleration of the respiration appearing after infection. A decrease of the % stimulation does not necessarily point to a decrease of the DNP-effect and the degree of limitation of the respiration by the phosphorylation, as a higher initial respiration rate must show a greater increase of the O_2-uptake to obtain an equal % stimulation. Consequently it is difficult to draw conclusions from the percentages. Therefore, in the following discussion the stimulation calculated in μl. O_2 will be compared.

The decrease of the DNP-effect in μl. O_2 indicates that the rate-limiting action of the phosphorylation was smaller after an increase of the respiration due to infection. In sample inf. 1 of expt. IV, cut on the border between the soft and the apparently normal tissue, this limitation would be absent.

Within the various tuber halves the O_2-uptake of the samples inf. 3–5 before addition of DNP was not equal. Yet it was stimulated by DNP to about the same level, especially in the expts. III and IV. This suggests that DNP accelerated the respiration, till a factor which had not changed under the influence of the infection became rate-limiting for the respiration. As it is generally assumed that DNP accelerates or uncouples the phosphorylation, and as the DNP-effect decreased with the increase of the respiration after infection, these observations might be interpreted as an indication that, at least initially, the phosphate-acceptor regeneration, which regulates the respiration rate, is accelerated under the influence of the infection without a change of other factors which may become rate-limiting in consequence of this acceleration.

With the exception of sample inf. 1 of expt. IV, the O_2-uptake of the samples inf. 1 and 2, which was already markedly increased after infection, reached higher values after addition of DNP than the O_2-uptake of samples not influenced by the infection did. The latter values of O_2-uptake remained lower than (expt. II) or hardly increased to (expts. III and IV) the values of the samples with the greatest respiratory increase before addition of DNP. So with continued influence of the infection more changes take place. Possibly this might

be connected with an increase of the intensity and/or capacity of the processes which may become rate-limiting after acceleration of the phosphorylation, or/and with the development of a pathway causing an extra O_2-absorption which is not regulated by the rate of phosphorylation.

In DNP-milieu the O_2-uptake of the samples without and with a slight respiratory increase after infection (inf. 3–5) was equal, especially in the expts. III and IV. This makes it unlikely that already in the initial stages of respiratory stimulation after infection some oxygen was taken up as a result of the development of such a pathway.

The above reasoning is based on the assumption that DNP accelerates or uncouples the phosphorylation to such a degree that it is no longer rate-limiting for respiration. Now HONDA (1956) already remarked that possibly DNP alone is not suitable for determining the exact degree with which the coupled phosphorylation is rate-limiting. On the grounds of experiments with preparations of cauliflower-mitochondria LATIES (1953) also mentioned this possibility. When α-ketoglutarate was oxidized by these preparations, a much greater O_2-uptake was observed after addition of a sufficient quantity of hexokinase and glucose than when the regeneration of phosphate-acceptor sites was accelerated by DNP. These experiments *in vitro* suggest that the metabolic mechanisms are more effective than DNP in liberating these sites. Consequently, in intact tissue too the O_2-uptake after stimulation by DNP may be considerably lower than in tissue in which the phosphorylation is no longer rate-limiting for the respiration due to a complete uncoupling or an accelerated phosphate-acceptor regeneration. The differences in O_2-uptake remaining after the action of DNP and amounting to resp. 82, 35 and 40 μl. O_2 in the expts. II–IV (column B: inf. 1–4; expt. IV inf. 2–5), possibly have to be attributed to a stimulation of the respiration by DNP, without the phosphorylation ceasing to be rate-limiting, and to a more efficient phosphate-acceptor regeneration by the endogenous processes as compared with the regeneration after addition of DNP.

As appears from table 16 (column C), a considerably increased O_2-uptake was still stimulated by DNP, although sometimes the effect was very slight. So DNP was capable of increasing a high respiration rate. Consequently, if DNP *in vivo* in potato-tuber tissue only accelerates or uncouples the phosphorylation to such a degree that the phosphate-acceptor regeneration remains rate-limiting, it is not to be expected that the effect of DNP on a respiration which has slightly increased after infection, will be considerably smaller than the effect on a normal respiration.

Now a considerable decrease of the DNP-effect was observed indeed. Moreover, within each tuber half the O_2-uptake of the samples with a slight respiratory increase after infection or with a normal respiration rate was increased by DNP to the same level. Therefore, it is more likely that DNP completely abolishes the limitation of the respiration rate by the phosphate-acceptor regeneration and stimulates the O_2-uptake to a rate at which other factors become limiting.

From the preceding discussion the following picture is obtained. The respiration rate of healthy tissue is limited by the coupled phosphorylation, probably by the activity of the transphosphorylating system and the regeneration of phosphate-acceptor sites. When the respiration increases after infection, the phosphate-acceptor regeneration becomes less limiting, even when the respiratory increase is slight. One observation made in tissue cut from the border between the soft and the apparently normal tissue (expt. IV inf. 1), the O_2-absorption of which was not enlarged by DNP, indicates that in such tissue the phosphorylation is no longer rate-limiting.

It is likely that no pathway for an O_2-uptake uncontrolled by the phosphorylation, developed during the initial stages of respiratory increase after infection. In later stages this may indeed be the case, or the intensity and/or capacity of the processes which may become rate-limiting after acceleration of the phosphate-acceptor regeneration, may have increased.

Now the hypothesis is put forward that after infection of potato-tuber tissue the regeneration of phosphate-acceptor sites is accelerated, in consequence of which the respiration rate can increase, while probably also the intensity and/or capacity of other processes which may become rate-limiting after acceleration of the phosphorylation, increases with continued influence of the infection. It is possible that in the later stages of respiratory increase after infection the respiratory pathway does change qualitatively. This would be in agreement with the experiments with malonate (Chapter III), in which a decrease of the % inhibition was observed in apparently normal tissue which had been strongly influenced by the infection.

CHAPTER VI

CHANGES IN PHOSPHATE COMPOSITION AFTER INFECTION

1. Introduction

If the hypothesis is correct that respiration is stimulated after infection because of an accelerated regeneration of phosphate-acceptor sites, in tissue with increased O_2-absorption the consumption of high-energy phosphate ($\sim$P) will be enhanced. This may be achieved either by direct breakdown of these $\sim$P-compounds or by stimulation of energy-requiring synthetic processes. The phosphate groups are liberated as inorganic phosphate or incorporated in organic compounds.

In experiments with sweet potato (Akazawa and Uritani 1956) and potato tubers (Akazawa 1956a) Akazawa and Uritani compared sound tissue adjacent to parts infected with *Ceratostomella fimbriata* with non-infected tissue. Some evidence was provided in support of a stimulation of synthetic processes after infection. In ethanol-$HClO_4$ extracts of tissue homogenates from both objects an increase of the acid-soluble organic phosphate fraction and the acid-insoluble nitrogen

fraction with concomitant decrease of respectively the inorganic phosphate and the acid-soluble nitrogen was reported after infection. The changes in the phosphate composition and the acceleration of the respiration by the disease were less considerable in the potato than in the sweet potato. AKAZAWA (1956b) observed a greater O_2-uptake per unity of mitochondrial nitrogen in mitrochondria preparations from infected sweet potato oxidizing α-ketoglutarate. A greater activity of ATP-ase and of acid and alkaline phosphatase was also reported. Authors think it possible that the accelerated respiration after infection is accompanied by an increase of the activity of various enzymes and of the synthesis of functional proteins, possibly also of enzyme proteins. The observation of AKAZAWA, UMEMURA and URITANI (1957) that the electrophoretic picture of protein extracts obtained from sound tissue adjacent to the infected parts differs from that of healthy sweet potato tissue, seems to support this view.

In this chapter experiments will be described that were undertaken in order to see whether after infection with *Gibberella saubinetii* (*Mont.*) *Sacc.* changes are to be found in the composition of the phosphate fraction of potato-tuber tissue in relation to the respiratory stimulation. In addition to the estimation of total phosphate and free inorganic phosphate attention will be paid to the easily hydrolysable organic phosphate and the norit-adsorbed phosphate. According to CRANE and LIPMANN (1953) and BORST PAUWELS (1956) in acid solution chiefly the nucleotides are adsorbed to norit, whereas inorganic phosphate and the hexosephosphates are not adsorbed.

2. METHODS

In the experiments described in this chapter only pyrex glass equipment and pro analyse chemicals (Merck) were used.

a. *Tissue extracts*

The tissue was killed in 5 ml. ethanol 96 % by heating to 80° C. for 5–10 min. After homogenization in 3 ml. ethanol 96 % the homogenate was quantitatively transferred to a centrifuging tube and after 15 min. extraction at room temperature centrifuged at 0° C. The supernatant was added to the ethanol in which the disks had been killed. The residue was resuspended in 2 ml. distilled water and centrifuged after 15 min. This was repeated four times. The supernatants were added to the ethanol extract. As ethanol interfered with the phosphate estimations, all ethanol was removed by concentrating the extract to less than 5 ml., after which the pH was adjusted to 4.0–4.2 with acetate buffer pH 4.2 (equal volumina 1 N. acetic acid and 0.025 N. sodium acetate). Now the extract was ready for use.

b. *Estimation of inorganic phosphate*

Inorganic phosphate was estimated in extracts using the colorimetric method according to LOWRY and LOPEZ (1946). In a Unicam Spectrophotometer (glass absorption cell 3.5 ml., light path 10 mm.) the intensity of the developing colour was measured at 5 min. intervals at 800 mμ., till it was constant. In each series a blank was included. The amount of phosphate was calculated in μg. phosphorus.

c. *Estimation of total phosphate in tissue disks*

Destruction of the tissue in sulfuric acid at high temperature liberates all phosphate as inorganic phosphate. In a pyrex glass tube with 2 ml. 10 N. H_2SO_4 five potato

disks were heated to about 160° C. on a sand bath. After some hours 2 drops of a 30 % H_2O_2-solution were added to accelerate the discoloration of the liquid and the destruction was continued. This was repeated every hour, until a complete discoloration was obtained. After cooling, 1 N. sodium acetate was added till the pH was 4.0, and then the mixture was diluted with acetate buffer pH 4.2. Now the inorganic phosphate could be estimated.

As an excess of H_2O_2 inhibits the colour formation with the molybdate-ascorbic acid reagent, only so much H_2O_2 was added during the destruction as was strictly necessary for complete discoloration.

d. *Estimation of easily hydrolysable phosphate*

The inorganic phosphate, liberated in the extracts by a short hydrolysis in 1 N. HC1 at 100° C., was estimated after heating tubes containing 2 ml. extract mixed with 2 ml. 2 N. HCl in a boiling water bath during 8 min. After cooling and adjusting the pH to 4.0 with 1 N. sodium acetate, the inorganic phosphate was determined, if necessary, after dilution with acetate buffer pH 4.2.

e. *Estimation of norit-adsorbed phosphate and not-adsorbed phosphate*

Before use norit SX II was freed from interfering substances. A suspension in 10 % trichloroacetic acid (TCA) was boiled for 20 min., filtered and the norit thoroughly washed with distilled water.

From an extract a 5 ml. sample was acidified with 2 ml. 10 % TCA and mixed well with 300 mg. norit. This was shaken a few times. After 30 min. the norit was centrifuged. In order to remove the not-adsorbed phosphate, the norit was twice resuspended in 3 ml. acetate buffer pH 4.2 and centrifuged after 15 min. The supernatants were mixed and the pH was adjusted to 4.0 with 1 N. sodium acetate and the inorganic and easily hydrolysable phosphate was estimated. The amount of easily hydrolysable phosphate adsorbed to the norit was determined as inorganic phosphate, liberated by suspending the norit in 4 ml. 1 N. HCl and heating to 100° C. during 8 min. The pH of the liquid was adjusted to 4.0 with 1 N. sodium acetate, the norit centrifuged and the supernatant diluted, if necessary, with acetate buffer pH 4.2.

The methods described were tested with a solution of adenosinediphosphate (ADP) containg 54 μg. phosphorus per ml. and with a potato-tissue extract. In table 17 the results are presented of estimations in the untreated ADP-solution (1–3) and after adsorption to norit in the supernatant (4–5) and the adsorbed fraction (6–7). The total phosphate found agreed with the calculated amount. In the solutions a small part of the ADP appeared to be hydrolysed (2). The ADP was completely adsorbed to norit (5). Only the terminal phosphate group of ADP is easily hydrolysable (UMBREIT c.s. 1951). As might be expected, about half of the adsorbed phosphorus was liberated by weak hydrolysis (6–7). From the agreement of the values of columns 2 and 4 and of columns 3 and 6 we may conclude that the norit-method

TABEL 17. Phosphate fractions in μg. phosphorus/ml. of a solution of adenosinediphosphate (ADP) containing 54 μg. P/ml. (pH 4.0).

untreated ADP-solution			after adsorption with norit			
			supernatant		norit	
total P (1)	inorganic P (2)	easily hydrol. P (3)	inorg. P (4)	easily hydrol. P (5)	easily hydrol. P (6)	not-easily hydrol. P (7)
55 μg.	5 μg.	26 μg.	5 μg.	0 μg.	27 μg.	24 μg.

is suitable. Identical results were obtained with 200 mg., 300 mg. and 500 mg. norit per 5 ml. solution. In the experiments 300 mg. norit was used.

From the potato extract only a slight fraction was adsorbed to norit. From table 18 it appears that similar values were obtained with parallel extracts from tissue of the same tuber. The agreement of the values obtained with and without norit treatment for both inorganic phosphate (1 and 3) and easily hydrolysable phosphate (2 and 4+5) shows that also with extracts the norit-method may be employed. What compounds are adsorbed was not investigated.

TABLE 18. Phosphate fractions in μg. phosphorus/20 disks in two parallel extracts from each 20 disks of one tuber.

extracts	untreated extracts		after adsorption with 300 mg. norit		
			supernatant		norit
	μg. inorganic P (1)	μg. easily hydrolysable P (2)	μg. inorganic P (3)	μg. easily hydrolysable P (4)	μg. easily hydrolysable P (5)
I	67	17	65	7	9
II	64	17	63	7	9

3. Experimental results

In discussing the changes in the phosphate composition of tissues with a respiration stimulated after infection, it is of importance to study whether the parasitic fungus, which for its nutrition depends on the tissue constituents, withdraws phosphorus-containing compounds from non-invaded cells.

The samples were analysed after measuring the O_2-uptake during two one hour intervals. The amounts of phosphate were calculated in μg. phosphorus per gram of tissue (fresh weight).

From the experiments it appears that a distinction should be made between tuber halves in which a zone with soft tissue has developed after infection, and halves in which this is not so. The values of table 19 show that when no soft tissue occurs, the content of total phosphate (2) in the samples of a tuber half is the same. Consequently, there is no question of a withdrawal of phosphorus-containing compounds from non-invaded cells by the pathogen. The amount of inorganic phosphate (3) distinctly decreases with increased stimulation of the respiration. After infection the organic phosphate (4), calculated as the difference between total phosphate and inorganic phosphate, increases with concomitant acceleration of the respiration.

In samples from infected tuber halves provided with a zone of soft tissue changes in total phosphate and inorganic phosphate are negligible, except in the tissue (inf. 1) situated on the border between the soft and the apparently normal tissue, the inorganic phosphate content of which is distinctly lower. However, the total phosphate also decreases.

TABLE 19. Total phosphate content and the amount of inorganic phosphate in relation to the respiration rate estimated in samples from non-infected tuber halves and infected halves with and without a zone with soft tissue after incubation at 25° C. Organic phosphate calculated from total and inorganic phosphate. Values averaged for 3 experiments. Phosphate in μg. P/gram (fresh weight).

tissue zones		% respiratory stimulation (1)	μg. total P (2)	μg. inorganic P (3)	μg. organic P (calculated) (4)
non-infected		0	485	102	383
infected halves without soft tissue	infected 1	170	522	57	465
	infected 2	71	517	73	444
	infected 3	21	529	92	437
	infected 4	0	522	95	427
infected halves with soft tissue	infected 1	*18*	*479*	*71*	*408*
	infected 2	50	558	110	448
	infected 3	21	567	115	452
	infected 4	0	590	120	470

By the estimation of the easily hydrolysable phosphate and the norit-adsorbed fraction of this an attempt was made to obtain further information on the nature of the organic phosphate fraction that increases after infection, when no soft tissue has developed. From table 20 it is clear that no increase of these fractions was observed in infected tuber halves. On the contrary, the norit-adsorbed, easily hydrolysable phosphate tends to decrease at higher respiration rates. The reality of these differences, however, is questionable in view of the slight amount of phosphate of this fraction and the accuracy of the method of estimation. In tuber halves with a zone of soft tissue the

TABLE 20. Fractions of easily hydrolysable phosphate in relation to the respiration rate estimated in samples from non-infected tuber halves and infected halves with and without a zone with soft tissue after incubation at 25° C. Zones inf. 1 nearest to the surface. Values averaged for 3 experiments, in infected halves with soft tissue averaged for 2 experiments. Phosphate in μg. P/gram (fresh weight).

tissue zones		% respiratory stimulation	μg. easily hydrolysable P	μg. norit-adsorbed easily hydrolysable P
non-infected		0	36	16
infected halves without soft tissue	infected 1	87	31	17
	infected 2	30	34	18
	infected 3	11	33	21
	infected 4	0	27	22
infected halves with soft tissue	infected 1	*12*	*11*	*9*
	infected 2	33	26	18
	infected 3	16	23	20
	infected 4	0	22	22

same is true, except that the decrease of both fractions in the tissue situated on the border between the soft and the apparently normal tissue (inf. 1) seems to be real indeed.

4. Discussion

For its nutrition the parasitically growing fungus is dependent on the constituents of the host tissue. Indeed, in cells of the soft tissue from infected potato-tuber halves a decrease of all estimated phosphate fractions was observed, indicating a general decrease of the phosphate content. On the other hand it appeared clearly from the experiments that no phosphorus-containing compounds had been withdrawn from non-invaded cells of the potato tuber, as far as these cells are apparently normal and not situated in a zone with soft tissue. Consequently, changes in the phosphate composition of apparently normal, non-invaded cells should be understood as internal shiftings that occur simultaneously with the respiratory reaction to the infection.

From the stimulation of the O_2-uptake of healthy potato tissue by added DNP it was concluded that the coupled oxidative phosphorylation is likely to be rate-limiting for the normal respiration. As the somewhat increased O_2-absorption in 1/15 M. phosphate buffer was considered to be a salt respiration, the conclusion was drawn that the rate of phosphorylation in turn is probably not limited by the amount of inorganic phosphate in the cells. The observation that in infected tuber halves without a zone with soft tissue the amount of inorganic phosphate in the samples distinctly decreased with concomitant acceleration of the respiration, supports this view.

Therefore it is most likely that the normal respiration of potato-tuber tissue is limited by the amount and/or the regeneration rate of phosphate-acceptor sites, the latter depending on the dephosphorylation of the phosphorylated acceptors, either by direct breakdown leading to the formation of free inorganic phosphate or by the activity of the transphosphorylating system. The increase of the amount of organically bound phosphate at the cost of the inorganic phosphate which was demonstrated parallel to the acceleration of the respiration, points to the latter possibility, and may be interpreted as evidence in support of the hypothesis that the synthetic activity in the cells is enhanced. As the fractions of easily hydrolysable phosphate show hardly any change, the conclusion may be drawn that the increase of the organic phosphate must be ascribed to the formation of phosphate compounds from which the phosphate can not easily be hydrolysed.

However, the increase of organic phosphate does not always occur when the respiration is stimulated. In tuber halves in which after infection a zone with soft tissue has developed, the phosphate composition of cells outside the area with soft tissue does not seem to be affected or is only very slightly affected, and this is also found when the respiration has been relatively slightly stimulated at greater distance from the surface of infected tuber halves without soft tissue. This is not necessarily in conflict with the proposed hypothesis on the regulating mechanism of the respiration rate. For it is possible 1° that

energy-requiring processes show an increased activity and cause an accelerated phosphate-acceptor regeneration by which just as much phosphate is liberated as is bound by the phosphorylation of the acceptor sites, or 2° that an increased synthesis of organic phosphate is accompanied by an accelerated breakdown of phosphate compounds by which the over-all organic phosphate fraction remains equal. However, the possibility that an accelerated regeneration of acceptor sites is caused by a direct breakdown of the phosphorylated acceptor(s) can not be precluded.

In conclusion, the results of the phosphate analyses of disks from infected tuber halves without soft tissue are in support of the hypothesis that the respiration rate in non-invaded cells can increase after infection by an acceleration of the regeneration of phosphate-acceptor sites. If after infection a zone with soft tissue has developed, or if the respiration rate is relatively slightly stimulated, the results for non-invaded, not-soft cells are not in conflict with this hypothesis, but they do not support it either.

[CHAPTER VII

SOME EXPERIMENTS WITH P^{32}-LABELLED PHOSPHATE

1. Introduction

From literature it is known that inorganic orthophosphate can be taken up by potato-tuber tissue and incorporated into organic compounds (Loughman 1957, Calo and Varner 1957, Lundegårdh 1958a, b). In the following experiments with the radioactive phosphorus-isotope P^{32} an attempt will be made to obtain direct data concerning the phosphate metabolism of tissue affected by the parasitically growing fungus in comparison with that of normal tissue.

It is assumed that the isotopes P^{31} and P^{32} are chemically identical, and are taken up and metabolised by the cells at the same rate, without the radioactive radiation affecting the metabolism.

2. Material and methods

Carrier-free radioactive phosphate was obtained from the N.V. Philips Duphar as a 10^{-3} M. solution of $Na_2HP^{32}O_4$. Before use this solution was brought to an activity of 1 mC. per ml. by addition of 10^{-3} M. inactive Na_2HPO_4. It was confirmed by paper-chromatography that all P^{32} was in the form of inorganic orthophosphate.

Samples of 20 disks were prepared from successive zones of the infected potato-tuber halves. These samples were submersed separately in 1.5 ml. of a solution of pH 6.0, containing 1.5 mC. P^{32} and placed at 25° C. during 1.5 hours. The phosphate uptake was terminated by removing the disks from the medium, after which they were washed thoroughly 6 times with water and homogenised in 1 ml. ethanol 96 %. The homogenate was quantitatively transferred to a centrifuging tube and centrifuged after 30 min. The tissue residue was

resuspended in 1 ml. water and centrifuged again after 30 min. This was repeated once more. The extract of each sample was made up to 4 ml.

The radioactivity of the extracts was measured, after which chromatograms were prepared with a total activity of 2–5.10^5 counts/min. Strips (6 × 56 cm.) of acid-washed paper (Schleicher and Schüll 2043b Mgl. ausgew.) with at the start over the full width of the paper 0.1–0.4 ml. extract, dependent on the radioactivity per 0.1 ml., were equilibrated in closed glass jars at 21° C. and irrigated for about 70 hours with propanol-ammonia-water (60:30:10; v/v/v) in which 1 $^0/_{00}$ EDTA (disodium-ethylene-diamine-tetraacetate) was solved. The descendent technique was used. After drying in warm air, a strip of 2.8 cm. width was cut out in the length from the middle of the chromatogram and the radiation of each zone of 0.5 cm. was counted. The same strips were used for autoradiography on Kodak X-ray (Kodirex) film. The exposure-time was 3–4 days.

The radiation intensity was counted at 600 Volt with a mica-window Geiger-Müller tube-counter, 3 cm. in diameter (plateau region 500–800 Volt). The counter tube was fixed at 6 mm. distance from the paperstrip under a 3 mm. thick lead plate with a diaphragm 0.5 × 3 cm. As P^{32} is a β-particle emitter, it was not necessary to cut the 0.5 cm. zones from the strips, provided these were pressed against the diaphragm while counting. The "shadow-radiation" from adjacent zones was absorbed in the lead. When the activity/zone was high, an aluminium filter, which was ascertained to absorb 50 % of the radiation, was placed between the strip and the counter tube.

The radiation in counts/min. was corrected for the resolving time of the tube (dead time) and the background radiation, and was plotted in a diagram. The radioactivity of the various radiation peaks was calculated and corrected for the decay since the killing of the cells by homogenisation in ethanol.

3. Experimental results and discussion

A detailed discussion of the results is only possible when the various radiation peaks have been identified. However, a chromatographic identification of P-compounds is difficult and uncertain (Borst Pauwels 1956). In order to obtain at least some information concerning the position of the various compounds, a mixture of known phosphate esters was separated, using the method described above. Potato-tissue extract appeared to have no influence on the separation of the esters and on their position in the chromatogram. The compounds were localized by means of the molybdate spray reagent according to Bandurski and Axelrod (1951). In addition the nucleotides ATP and ADP were detected by making an ultraviolet print of the chromatogram with a Philips TUV fluorescent tube as a light source. As the solvent front had run from the paper, the position of the compounds was calculated with respect to the position of inorganic phosphate (Rp-values). The hexosephosphates glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate and the 3-phosphoglyceric acid ran faster than inorganic phosphate (Rp 1.12–1.39), whereas fructose-1,6-diphosphate was slower (Rp 0.54). ATP had a Rp 0.74. ADP ran about as fast as inorganic phosphate (Rp ± 1.0).

The concentration of the phosphate esters in the potato-tissue extracts was so low that they could not be detected either with the spray reagent or by UV-photography. Only inorganic phosphate was detected.

When a chromatogram of an extract from a P^{32}-treated sample was prepared, a number of radiation peaks were found in addition to

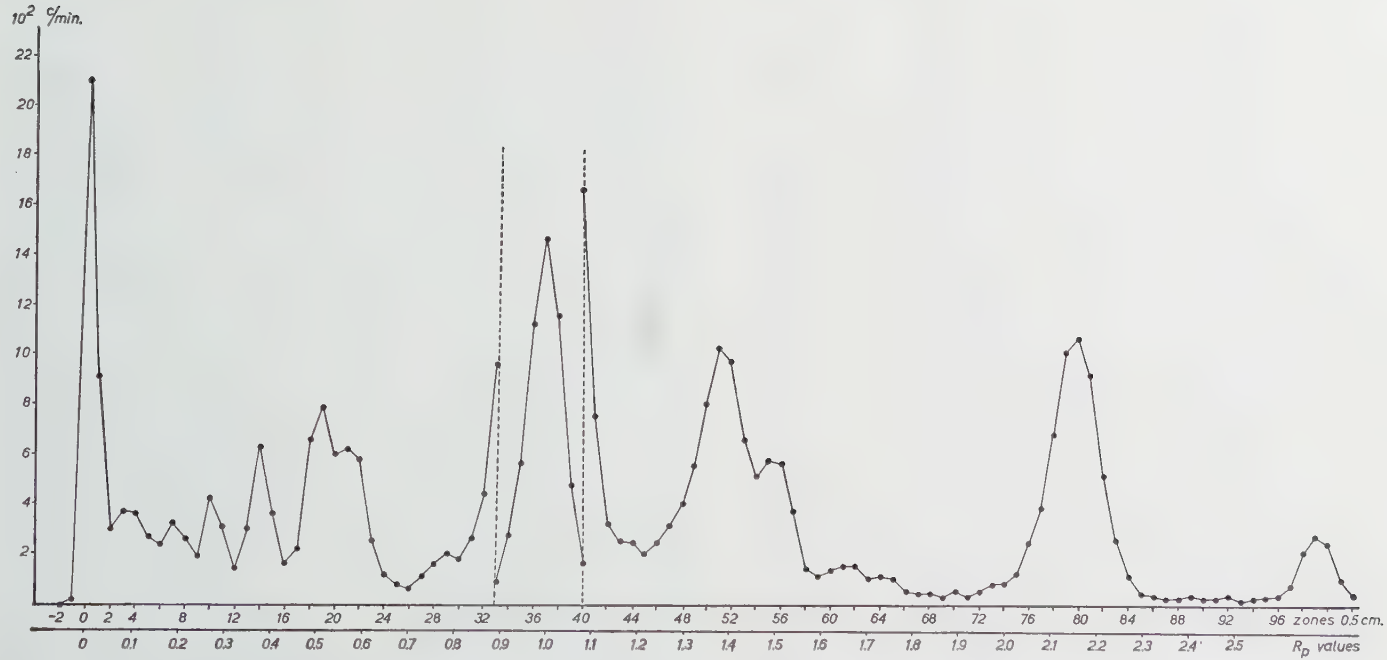

Fig. 8. Distribution of P^{32} on a chromatogram of extract from tissue disks cut near the region invaded by the fungus. Values corrected for dead time and background radiation. Of the inorganic phosphate peak the values of the zones 33–40 are drawn at scale 1/10. Total radioactivity of the paperstrip about 85,000 c./min.

inorganic phosphate. The fastest running peak observed had a Rp-value of about 2.7. This is illustrated by Fig. 8. After chromatographic separation of a norit-treated part of such an extract, the peaks with Rp < 1.0, except that with Rp 0.54 (fructose-1,6-diphosphate?), appeared to be absent, whereas in comparison with the untreated part the decrease of the compound with Rp 0.54, inorganic phosphate and the faster running compounds was negligible.

On account of the preceding data and the often incomplete separation of the peaks, the P^{32}-distribution in the extracts was calculated for some groups of compounds instead of for each peak separately. It was supposed that the compounds with Rp < 1.0, except peak Rp 0.54, are mainly nucleotides and that in the region Rp 1.10–1.50 chiefly hexosephosphates are localized. This is in agreement with the experience of BORST PAUWELS (1956). The compound with Rp 0.54 is probably fructose-1,6-diphosphate, and was included in the group "hexosephosphates".

Using the inorganic phosphate content in potato-tuber tissue as given in Chapter VI (table 19) and the radioactivity of the extracts obtained in the experiments with P^{32}, it could be calculated that the total amount of inactive and labelled phosphate taken up during the experimental period had hardly any effect on the concentration of inorganic phosphate in the cells. This concentration will have increased by at most a few %. Consequently, it may be assumed that at a certain concentration of inorganic phosphate in the cells the amount of organically bound P^{32}-labelled phosphate, which is dependent on the concentration-ratio P/P^{32}, is more or less proportional to the amount of P^{32}-phosphate taken up.

From the radioactivity of the extracts it appeared that the uptake of P^{32}-phosphate by tissue adjacent to the area invaded by the fungus, was greater than the uptake by tissue at a greater distance. This may be related to the increased respiration rate after infection, and then this is in accordance with the observations of LUNDEGÅRDH (1958a, b) according to which the entrance of organic phosphate into cells of potato-tuber tissue after a rapid initial absorption depends on the metabolic activity of the cells.

As a consequence of the differences in uptake, the absolute amounts of organically bound P^{32}-phosphate in counts/min./unity of extract can not be used as a measure for the activity of the phosphate metabolism in the cells. Therefore, the amounts of P^{32} in the compounds were calculated from the distribution of the radiation on the chromatograms as % of the total activity of the paperstrip, that is as % of the ethanol-water-soluble labelled phosphate.

In table 21 the P^{32}-distribution over the groups of "nucleotides", inorganic phosphate, "hexosephosphates" and compounds with Rp-values > 1.50 is given for extracts of tissue from five successive zones of infected tuber halves. The % are average values of 4 experiments. For comparison the values for non-infected tissue are also given.

Although a detailed quantitative comparison of the P^{32}-distribution

in the various extracts is impossible, yet some general features find expression in table 21. The P^{32}-distribution over the groups of compounds was nearly the same in extracts from non-infected tissue and from tissue of the infected halves at a great distance from the zone with mycelium. However, within the infected tuber halves marked differences occurred in the % "nucleotides" and "hexosephosphates". These percentages increased, resp. decreased with increasing influence

TABLE 21. Distribution of P^{32} in extracts from tissue of infected and non-infected tuber halves incubated at 25° C. Zones inf. 1 nearest to the surface. % mean values for 4 experiments.

fractions	non-infected	inf. 1	inf. 2	inf. 3	inf. 4	inf. 5
"nucleotides"	3.0 %	7.7 %	5.1 %	3.9 %	3.3 %	2.9 %
inorganic phosphate	73.9 %	72.8 %	72.5 %	71.9 %	72.9 %	71.4 %
"hexosephosphates"	13.3 %	9.7 %	12.2 %	13.8 %	14.2 %	15.2 %
Rp > 1.50	8.1 %	8.9 %	8.7 %	8.9 %	8.4 %	9.3 %
Total	98.3 %	99.1 %	98.5 %	98.5 %	98.8 %	98.8 %

of the disease. As it was not ascertained whether the P/P^{32} ratio in the various compounds had become constant at the end of a 1.5 hours experimental period, differences in % may indicate either differences in the amount of the compounds or differences in the rate of conversion.

The influence of the differences in phosphate uptake on the P/P^{32} ratio in the cells induced us to calculate the P^{32}-distribution in % of the total radioactivity of the soluble P^{32}-fraction. Now the P/P^{32} ratio is not only determined by the uptake of phosphate but also by the inorganic phosphate content in the cells at the start of the P^{32}-treatment. Table 19 in Chapter VI showed that this content may decrease under the influence of the infection. However, a decrease by more than about 40 % was never found.

Starting from this greatest decrease of the inorganic phosphate content, it might be supposed that in the experiments with P^{32} this content in the samples inf. 1 was 40 % lower than in the samples inf. 5. If this is true, and if the rate of incorporation of inorganic phosphate was the same in all samples of a tuber half, it might be expected that the % P^{32} in the various groups of organic phosphate compounds should be 10/6 times higher in inf. 1 than in inf. 5. According to table 21 the average % P^{32} in the "nucleotides" of the samples inf. 1 was 2.5 times that of the samples inf. 5. When the % of inf. 1 is corrected for the increase with respect to the % of inf. 5 which may be expected after this decrease of the inorganic phosphate content after infection, the % P^{32} in the "nucleotides" in inf. 1 still remains 1.5 times as high as in inf. 5.

The amount of P^{32} incorporated in the in ethanol-water-insoluble compounds was not determined in the experiments and was not included in the calculation of the % of table 21. If this fraction is taken into account, the % of table 21 should be corrected by multi-

plying the % by the factor $\frac{\text{soluble } P^{32}}{\text{soluble} + \text{insoluble } P^{32}}$. If the % P^{32} in the insoluble fraction of the samples of each infected tuber half (inf. 1–5) was the same, this factor will be equal within a tuber half, and the ratio of the % P^{32} in each group of compounds will remain equal to the ratio without applying this correction. If, however, the % P^{32} in the insoluble fraction of the samples near the area with mycelium was greater than it was in the tissue at greater distance from the infection-surface, the factor will decrease when approaching the surface. In that case the differences in the % P^{32} in the "nucleotides" will be relatively reduced as a result of the correction.

So the increase of the % P^{32} in the "nucleotides", which might be interpreted as an indication that the rate of conversion and/or the amount of these compounds had increased under the influence of the infection, might also be explained in another way. Only a part of the increase of the % in inf. 1 as compared with the % in inf. 5 might be attributed to the possible decrease of the inorganic phosphate content in the cells by at most 40 % (Chapter VI) under the influence of the infection. That the ethanol-water-insoluble P^{32}-fraction was not included in the calculation of the % of table 21, may only be put forward to explain the difference in the % P^{32} in the "nucleotides" or a part of it, if it is assumed that the % P^{32} in the insoluble fraction increased with increasing influence of the infection. The latter assumption suggests an increase in the amount of the compounds of this fraction and/or of their rate of conversion. Assuming that the inorganic phosphate content in the samples inf. 1 had decreased to 60 % of the content in the samples inf. 5 (the largest decrease observed in Chapter VI) and that the higher % P^{32} in the "nucleotides" does not indicate an increase of the amount and/or the rate of conversion of these compounds, the correction-factor $\frac{\text{soluble } P^{32}}{\text{soluble} + \text{insoluble } P^{32}}$ should be 1.5 times as high in the samples inf. 5 as in the samples inf. 1. This implies that the insoluble P^{32}-fraction in inf. 1 should have been at least more than half of the soluble P^{32}-fraction or a third of the total amount of the P^{32} taken up.

Anyhow, it may be concluded that the increase of the % P^{32} in the "nucleotides", as found in table 21, is an indication for the increase of the amount and/or the rate of conversion of the "nucleotides" and/or of the compounds of the ethanol-water-insoluble P^{32}-fraction.

When the above corrections are also applied to the % of the compounds with $Rp > 1.50$ and of the "hexosephosphates", it appears that under the influence of the infection the % P^{32} in these groups of compounds decreased, resp. decreased even more than is indicated by table 21.

Especially the decrease after infection of the % P^{32} in the "hexosephosphates" is considerable. This suggests that the amount of these compounds and/or their rate of conversion in the samples inf. 1 was less than in the samples inf. 5. A lower rate of conversion of the hexosephosphates after infection seems unlikely, as in potato-tuber

tissue these compounds are said to be closely connected with the respiration and consequently it is more probable that an accelerated conversion of hexosephosphates will take place when the respiration is increased after infection. Therefore, it is more likely that the amount of these compounds decreased. This does not necessarily mean that the synthesis of hexosephosphates was retarded too. For, if with a higher respiration rate the consumption of hexosephosphates increases to a greater extent than the rate of synthesis, the amount of the compounds will decrease, even if the rate of synthesis is enhanced.

It is not known what compounds correspond with the radiation peaks with Rp > 1.50. So, about this group of compounds no further information can be given.

The general picture obtained from the experiments with P^{32} is that the amount and/or the rate of conversion of the "nucleotides" and/or possibly of the ethanol-water-insoluble P-containing compounds increased after infection. Of the organic phosphate-compounds it is especially the nucleotides that are closely connected with the metabolism of high-energy phosphate and the regeneration of phosphate-acceptor sites. So the P^{32}-distribution in the extracts of the samples inf. 1–5 indicates an accelerated formation of $\sim$P-bonds and phosphate-acceptor regeneration. If the amount of the "nucleotides" increased, a greater capacity of the $\sim$P-metabolism after infection is indicated. A possible increase of the insoluble P^{32}-fraction after infection would mean an enlarged formation of insoluble organic phosphate-compounds, and this enlarged formation might be accompanied by a greater consumption of high-energy phosphate and by an accelerated phosphate-acceptor regeneration.

The results of the experiments with P^{32}-labelled phosphate are considered to support the hypothesis that the regeneration of phosphate-acceptor sites is accelerated after infection, in consequence of which the respiration rate can increase.

CHAPTER VIII

GENERAL DISCUSSION

For the investigation of the reaction of the host cells to the infection it was necessary to separate the gas-exchange of the host from that of the parasite. Therefore, in the experiments with infected tuber halves tissue samples were cut from the area not penetrated by the fungus. What happened in the host cells invaded by the mycelium, was not investigated.

The question may be asked whether the respiratory increase observed in the mycelium-free tissue of the infected tuber halves, was a result of the infection with the fungus, or whether a wound-reaction which occurs after cutting the tubers into two halves before inoculation, played a more or less important part. For after incubation at 25° C. a respiratory increase was also observed in the non-infected tuber halves (table 1). This increase was restricted to a zone of 2–3 mm. immediately below the surface of the cut, which was blocked by

a cork layer, and was already at its maximum after a 24 hours' incubation (increase 80–90 %). In the infected tuber halves no cambial activity was ever observed under the places where the fungus was growing (Richter, unpublished). A blocking of the surface by the formation of a cork layer did not occur. Moreover, the respiratory increase in the mycelium-free tissue often proved to be much larger than 80–90 % (table 16), and often an increase of the O_2-uptake could still be demonstrated at a distance from the surface which was much greater than 2–3 mm. When the penetration of the mycelium into the tissue is taken into account, the distance to the zone with mycelium was still greater than 2–3 mm. The graphs reproduced in Fig. 2 and 3 further suggested that differences in intensity of infection and in the duration of the incubation at 25° C. corresponded to differences in the extent of the respiratory increase. Therefore it seems reasonable to conclude that the increase of the respiration in infected tuber halves was caused by the infection with the fungus. Yet it remains possible that a wound-reaction played a part, as the growing mycelium continually penetrated into new cells, which resulted in a continual wounding of cells. On the contrary, in the non-infected tuber halves cells were wounded only once. Whether compounds excreted by the fungus have a function in causing the respiratory reaction, has not yet been ascertained, although HELLINGA (1942) thought he had found evidence for this assumption.

Concerning the respiratory increase in the tissue adjacent to the zone with mycelium, according to the hypothesis submitted in Chapter V this increase is caused by an acceleration of the phosphate-acceptor regeneration. This would be in agreement with the decrease after infection of the stimulating DNP-effect. However, this decrease might also indicate a partial uncoupling of the respiration and the phosphorylation. Now, in the experiments with P^{32}-phosphate always 10–15 radiation peaks were found on the chromatograms also after a strong influence of the infection. From the P^{32}-distribution on the paperstrips it was concluded that the amount and/or the rate of conversion of the "nucleotides" and/or the insoluble P^{32}-containing organic phosphate had increased under the influence of the infection. By means of the phosphate analyses (Chapter VI) an increase of the organic phosphate was indeed found in infected tuber halves without a zone with soft tissue. As, in addition, the O_2-uptake of samples with greatly enhanced respiration was still stimulated by DNP, it seems unlikely that in the apparently normal tissue of infected tuber halves a complete or partial uncoupling occurred under the influence of the infection.

The O_2-uptake may be considerably enlarged after infection. It is not likely that a qualitative change of the respiratory pathway developed in the initial stages of respiratory increase. For in these stages no differences in % inhibition were found after addition of respiratory inhibitors, and the O_2-uptake of samples with a slightly increased respiration was stimulated by DNP to the same level as the O_2-uptake of samples with a normal respiration rate was. After addi-

tion of malonate only in the later stages of respiratory increase evidence was obtained for a qualitative change of the respiratory pathway.

When in an infected tuber half a zone with soft tissue had developed, not only the tissue-structure appeared to be affected in this area, but the cells had also lost their turgescence and the respiration rate in this tissue proved to be lower than that in the adjacent apparently normal tissue with increased respiration. This was accompanied by changes in the reaction shown by the O_2-uptake to addition of some inhibitors, whereas the O_2-uptake was not stimulated by DNP (one observation). So, when the tissue becomes soft, more changes take place than were observed in the apparently normal tissue. This might be connected with the dying of the cells.

SUMMARY

I

The respiratory increase observed in many infected plant parts might be partly or wholly attributed to the gas-exchange of the pathogen. With potato-tuber tissue infected with *Gibberella saubinetii* (*Mont.*) *Sacc.* investigations were made in order to find out whether a respiratory increase could be demonstrated in the host cells, and whether this increase was accompanied by a qualitative change of the respiratory pathway. In the second place the mechanism by which the respiration rate is regulated was investigated.

II

As the fungus only penetrates the outer cell layers (2–3 mm.) of potato-tuber tissue, the respiration of host cells which did not contain mycelium, could be investigated in the tissue next to the invaded parts. From the two halves of each tuber, one of which was inoculated on the surface of the cut while both were incubated at 25° C., tissue samples were cut at various distances from the surface of the original cut. In the infected halves the respiration was distinctly accelerated in the proximity of the area invaded by the fungus, and decreased with increasing distance to this area, to reach a nearly constant level in the zones at greater distance. Occasionally a zone with soft tissue was found close below the area with mycelium. The O_2-uptake of this soft tissue was lower than that of the adjacent apparently normal tissue, the respiration of which was accelerated. The results of many experiments suggested that in tuber halves without a zone with soft tissue differences in respiratory increase and in the distance from the surface where this increase could still be demonstrated, corresponded with differences in intensity of infection and in duration of the incubation at 25° C. (Fig. 2 and 3). In the non-infected halves an increased respiration was observed in a narrow area (2–3 mm.) immediately below the surface of the cut, which was blocked by a cork layer.

III

In order to find out whether the respiratory mechanism had changed qualitatively after infection, R.Q.-values were determined and the effect of respiratory inhibitors was examined in tissue from non-infected and infected tuber halves. The R.Q.-values of non-infected tissue and those of host cells which were not penetrated by the mycelium, were equal. The O_2-uptake of all samples from apparently normal tissue of the infected and the non-infected halves of the tubers was retarded to the same extent after addition of various inhibitors, namely with 90–95 % by 0.002 M. iodoacetate pH 6.2, with about 80 % by 0.02 M. sodium fluoride pH 6.2 and with 85–90 % by 0.002 M. sodium azide pH 6.2. In samples with a large increase of respiration after infection the inhibition by 0.06 M. malonate pH 4.3 proved to be lower (20–40 %) than in the other samples (65–75 %). Probably malonate did not attack succinic dehydrogenase exclusively.

When a zone with soft tissue developed after infection, the softening of the tissue was accompanied by a decrease of the respiration rate and its inhibition by iodo-

acetate and sodium fluoride. Data on the effect of the other inhibitors on the respiration of this tissue are not available.

For the apparently normal tissue without mycelium it was concluded that the experiments with malonate were the only ones which indicated a qualitative change of the respiratory pathway; this effect however, was noticed only after continued influence of the infection. In the zone with soft tissue changes in the pathway had developed which were not observed outside this area.

IV

In the infected tuber halves starch disappeared from the tissue adjacent to the area with mycelium. In this tissue the fructose content did not change. Occasionally a more or less parallel behaviour of the respiration and of the amounts of sucrose and/or glucose was observed. The changes in the sugar content after infection were considered to be a parallel phenomenon resulting from the general activation of the metabolism, which also caused the increase of the O_2-uptake, rather than the regulating mechanism of the respiration rate.

V

As the O_2-uptake of healthy potato-tuber tissue was markedly stimulated by 10^{-3} M. 2,4-dinitrophenol (DNP) pH 5.0, the respiration rate was supposed to be regulated by the coupled phosphorylation, which in its turn depends on the activity of the transphosphorylating systems and the regeneration of phosphate-acceptor sites. The stimulation by DNP of the O_2-uptake of apparently normal tissue from infected tuber halves decreased with the increase of the respiration after infection, which suggested a decrease of the rate-limiting action of the phosphorylation. Only in the tissue cut from the border between soft and apparently normal tissue the O_2-uptake was not increased by DNP, indicating that the respiration was no longer limited by the phosphorylation.

Although, especially in later stages of respiratory increase after infection, a qualitative change of the respiratory pathway could not be excluded, the hypothesis was put forward that in the apparently normal tissue of the infected tuber halves the regeneration of phosphate-acceptor sites was accelerated under the influence of the infection, in consequence of which the respiration rate could increase. Probably also the intensity and/or capacity of other processes which may become rate-limiting after acceleration of the phosphorylation increased with continued influence of the infection.

VI

Determinations of the total phosphate content showed that the fungus withdrew phosphorus-containing compounds from the non-invaded cells of soft tissue, not, however, from apparently normal tissue. In infected tuber halves without a zone with soft tissue a decrease of the inorganic phosphate and an increase of the organic phosphate was observed when respiration was markedly accelerated. In the apparently normal tissue of infected halves with soft tissue these two fractions were unchanged. In all infected halves the easily hydrolysable phosphate and its norit-adsorbed fraction tended to decrease with increasing respiration. Both fractions distinctly decreased in tissue cut from the border between soft and apparently normal tissue, which was probably a result of the withdrawal of phosphate-compounds by the fungus.

The increase of organic phosphate after infection suggested an enhanced synthetic activity in the host cells, which may cause an accelerated phosphate-acceptor regeneration. The absence of this increase of organic phosphate in tuber halves with a zone with soft tissue is not necessarily in conflict with this view.

VII

The influence of the infection on the esterification of inorganic phosphate was investigated using P^{32}-labelled phosphate ($Na_2HP^{32}O_4$). After the P^{32} taken up during 1.5 hours had been extracted with ethanol and water, the compounds n the extracts were separated by paper-chromatography. The distribution of the

radioactivity on the paperstrip was determined. The radiation of the "nucleotides" (Rp < 1, except Rp 0.54), inorganic phosphate (Rp 1.0), "hexosephosphates" (Rp 1.10–1.50) and the compounds with Rp > 1.50 was calculated in % of the total radioactivity of the paperstrips.

The % P^{32} in the "nucleotides" and the "hexosephosphates" appeared to increase, resp. decrease after infection. The influence exercised on the interpretation of these differences by a decrease after infection of the inorganic phosphate content in the host cells (Chapter VI), and by the fact that the P^{32}-fraction which is insoluble in ethanol-water, was not included in the calculation of the %, was discussed. It was concluded that the increase of the % P^{32} in the "nucleotides" is an indication for the increase of the amount and/or the rate of conversion of the "nucleotides" and/or of the compounds of the insoluble P^{32}-fraction. The decrease of the % P^{32} in the "hexosephosphates" does not necessarily mean that the synthesis and the rate of conversion of these compounds were retarded after infection. The results of the experiments with P^{32}-labelled phosphate were considered to support the hypothesis that after infection the regeneration of phosphate-acceptor sites was accelerated, in consequence of which the respiration was increased.

VIII

The rôle which the wound-reaction that occurred after the tubers were cut into two halves, may have played in the respiratory increase in infected tuber halves, was discussed. Although the respiratory increase in the infected halves was most likely a result of the infection, a wound-reaction caused by the continual wounding of cells by the penetrating mycelium, might have played a part.

From the results of the Chapters V, VI and VII the conclusion was drawn that it was not likely that in the apparently normal tissue of infected tuber halves an uncoupling of respiration and phosphorylation occurred. At least in the earlier stages of respiratory increase no evidence was obtained for a qualitative change of the respiratory pathway. In the soft tissue which developed in some infected tuber halves more changes were observed than in the apparently normal tissue. This might be connected with the dying of the cells.

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ÜBER DEN ENZYMATISCHEN CUTIN-ABBAU

I. MITTEILUNG: NACHWEIS EINES "CUTINASE"-SYSTEMS

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Einleitung

Wo pflanzliches Gewebe an die atmosphärische Luft angrenzt, tritt weit verbreitet eine Grenzhaut auf, die den Namen Kutikala trägt (Frey–Wyssling 1935). Sie ist ein morphologisch wohldefiniertes Gebilde, dessen chemischer Aufbau jedoch uneinheitlich ist. Neben den bekannten hochpolymeren Membranstoffen Cellulose, Pectin und Wachsen ist typisch das Vorkommen von *Cutin*, das dem in der Borke vorkommenden *Suberin* und dem Membranstoff der Pilzsporen und Pollenmembranen *Sporopollenin* verwandt ist. Erst in den letzten Jahren hat man den Versuch gemacht die chemische Konstitution des Cutins aufzuklären (vgl. Übersichtsreferat bei Van Overbeek 1956). Dabei zeigte sich, daß nicht nur die Dicke der Kutikularschicht sondern auch die Zusammensetzung der Kultikularstoffe stark variiert.

Die Kutikula stellt morphologisch und ökologisch eine Schutzeinrichtung dar. Der Abbau dieser Schutzschicht ist vor allem in Zusammenhang mit zwei biologischen Prozessen von großem Interesse:

a. beim Infektionsvorgang durch pathogene Pilze und Bakterien (vgl. Gäumann 1951),

b. bei der Inkompatibilitätsreaktion von Cruciferen:

nach Selbstbestäubung ist innerhalb dieser Familie die Erscheinung weit verbreitet, daß der Pollenschlauch nicht in der Lage ist, die Kutikula, welche die Narbenpapillen überzieht, zu durchbrechen (Tatebe 1939, Linskens 1955, 1959, Kroh 1956, Christ 1959).

Ohne einer chemischen Konstitution vorzugreifen wird unter dem Cutin vorläufig von uns verstanden: ein polymeres Kondensationsprodukt höherer Fettsäuren, das vor allem an der Grenzfläche Pflanzengewebe-atmosphärische Luft vorkommt und wesentlich Schutzfunktionen ausübt.

Literaturübersicht

Zusammensetzung der Kutikularschicht

Elektronenmikroskopische Untersuchungen der Kutikularschicht ergaben, daß die äußere Begrenzung durch Wachsplättchen gebildet wird, die die Größe von etwa 1–10 mμ haben (Mueller, Carr und Loomis 1954, Scott, Hamner, Baker und Bowler 1957). Das Wachs liegt als Film über der eigentlichen Kutikula, die aus einem Gerüst von Cellulose, Cutin und eventuell Pectin und Suberin besteht (Koljo 1957). Diese verschiedenen Substanzen können nach den Untersuchungen von Meyer (1938) mehr oder minder ausgebildete Schichten bilden. Die Kutikula reicht entweder direkt in die darunter liegende Celluloseschicht hinein (Roelofsen 1952), oder ist häufiger von dieser durch eine Pectinmembran getrennt (Anderson

1928, RAWLINS und TAKAHASHI 1952, ROELOFSEN 1952). Bei diesem einfachen Aufbau dient das Cutin der Kutikula nach der Auffassung von FREY–WYSSLING (1953) und HÄRTEL (1950) als Bindeglied zwischen der hydrophilen Cellulose und dem hydrophoben Wachs (vgl. KOLJO 1957). Der Aufbau der Kutikularschicht kann aber dadurch kompliziert werden, daß unter der eigentlichen Kutikula, durch eine Pectinmembran von dieser getrennt, eine breitere, cutinisierte Schicht liegt, auf die erst dann, wiederum durch eine Pectinmembran getrennt, die Celluloseschicht folgt (SITTE 1955, 1957). Innerhalb dieser cutinisierten Schicht finden sich häufig Wachseinlagerungen (SCOTT und LEWIS 1953, ESAU 1953), manchmal auch freie Fettsäuren (HILKENBÄUMER 1958), jedoch keine Cellulose (SITTE 1957). Durch die enge Bindung des Cutins an die übrigen Zellwandsubstanzen ist die Isolierung des Cutins erst relativ spät versucht, und seine chemische Zusammensetzung nur wenig untersucht worden. Reines Cutin wurde erstmals von LEGG und WHEELER (1925) erhalten und seine Zusammensetzung von LEGG und WHEELER (1929) und LEE (1925) näher untersucht. Nach diesen und anderen (vgl. Übersichtsreferat von MEARA 1955) chemischen Methoden zur Isolierung des Cutins von den übrigen Kutikularsubstanzen hat erst ORGELL (1955) eine biochemische Methode angegeben, die darauf beruht, daß die Pectinmembran zwischen Kutikula und Untergewebe mittels pectinspaltender Fermente aufgelöst wird. Den gleichen Erfolg bringt die Methode von SKOSS (1955), die darauf basiert, daß anaerobe Bakterien auf das Untersuchungsmaterial einwirken und alle Stoffe bis auf das Cutin abbauen, das auf diese Weise in reiner Form zurückbleibt.

Chemie des Cutins

Über die Chemie des Cutins war lange Zeit wenig bekannt. Erstmals extrahierte FRÉMY (1859, 1881, 1885) die Kutikula verschiedener Pflanzen mit Lösungsmitteln zur Entfernung der Wachse und behandelte den Rest mit kochendem alkoholischem Alkali. Dabei erhielt er das wasserunlösliche K-Salz des von ihm so benannten festen "Stearocutin" und das wasserlösliche K-Salz des halbflüssigen "Oleocutin". Später fand LEE (1925), daß die Kutikula von Chrysanthemen und Rosen sowohl veresterte, als auch freie Fettsäuren enthält und zur gleichen Zeit fanden LEGG und WHEELER (1925) freie Fettsäuren, Glycoside, verseifbares Material und Cellulose in der Kutikula von Blättern der Agave americana. Näheren Aufschluß über die Konstitution der Cutinfettsäuren brachten schließlich die Arbeiten von MATIC (1956 a, b), aus denen hervorgeht, daß mehr als 60 % des Cutins der Agave americana, in dem keine unverseifbaren Bestandteile gefunden wurden, aus vier verschiedenen Oxyfettsäuren bestehen, nämlich 9, 10, 18-Trihydroxy-octadecansäure, 10, 18-Dihydroxy-hexadecansäure, 10, 18-Hydroxy-octadecansäure und 10, 16-Dihydroxyhexadecansäure: 80 % dieses Fettsäuregemisches bestehen dabei aus der 10, 18-Dihydroxy-octadecansäure. Interessant ist dabei besonders, daß alle diese Fettsäuren endständige Oxygruppen besitzen. Es erscheint zweifelhaft, ob die in 9- oder 10-Stellung befindlichen Oxygruppen im Cutin tatsächlich enthalten sind, da bei der angewandten Methode eventuell vorliegende Doppelbindungen oxydiert werden können. Es ist nicht sicher, ob die genannten Verbindungen nicht zum Teil auch Artefakte darstellen, was auch von MATIC selbst (private Mitteilung 1959) für nicht ausgeschlossen gehalten wird.

Verestert man Fettsäuren mit derartigen endständigen Oxygruppen miteinander, so erhält man polymere Produkte, die unlöslich, elastisch und temperaturstabil sind, wie es beim Cutin der Fall ist. Cutin ist dem Suberin, das zur Hauptsache aus Phellonsäure ($C_{22}H_{44}O_3$) in Form von Polyestern besteht (DUPONT, DULOU und CHICOISNE 1956), nahe verwandt und ist auch wie dieses hochpolymer und vernetzt (ZETSCHE 1932, FREY–WYSSLING 1953, TREIBER 1955, 1957).

Entstehung des Cutins

Über die natürliche Synthese des Cutins ist nur wenig bekannt. Man nimmt an, daß die Cutinschicht durch Permeiren flüssiger Vorstufen des Cutins durch die bereits bestehende Cellulosewand und durch Polymerisation dieser Vorstufen an der Oberfläche unter Sauerstoffeinfluß gebildet wird (vgl. FRITZ 1935, PRIESTLEY 1943, LINSKENS 1950, 1952, SITTE 1955). Dafür spricht auch die neuerlich gemachte Beobachtung von BOLLIGER (1959), daß bei der Bildung der Kutikula von *Philodendron* elektronenmikroskopisch kleinste Fetttröpfchen nachweisbar sind, die von

innen her in Richtung der Oberfläche wandern. Bezüglich des möglichen Einflusses des Sauerstoffs auf die Cutinbildung sind die Ergebnisse von SIDDIQI und TAPPEL (1956) von Bedeutung: sie ließen ein Gemisch aus Leinöl und einer aus Erbsen gewonnenen Lipoxydase 7 Tage lang an der Luft stehen und erhielten dabei einen unlöslichen Film, der nicht nur äußerlich dem Cutin ähnelte, sondern auch die gleiche UV-Fluoreszenz hatte wie dieses. Zieht man diese mögliche Beteiligung der Lipoxydase an der Cutinbildung in Betracht, so kann man sich vorstellen, daß die Fettsäuren (Linol- und Linolensäure) zunächst innerhalb der Zelle zu Monohydroperoxyden oxydiert werden, die dann an die Oberfläche wandern, um dort (möglicherweise unter weiterer Sauerstoffaufnahme) zu Cutin zu polymerisieren.

Die Auffassung wird gestützt durch die Tatsache, daß sowohl autoxydativ (PRIVETT und NICKELL 1956, CHIPAULT 1952, SLOVER und DUGAN 1958) als auch enzymatisch (BERGSTRÖM 1945, FRANKE, MÖNCH, KIBAT und HAMM 1948, FRANKE und FREHSE 1953) anoxydierte Linolsäure starke Neigung zur Polymerisation zeigt. Nach den bisherigen Kenntnissen (vgl. KAUFMANN 1958) verläuft die Polymerisation über die Bildung konjugierter Hydroperoxyde, wobei in Abhängigkeit von der Temperatur entweder cis-trans- oder trans-trans-konjugierte Hydroperoxyde entstehen können (KHAN 1953, 1955, KHAN, LUNDBERG und HOLMAN 1954, PRIVETT, NICKELL, TOLBERG, PASCHKE, WHEELER und LUNDBERG 1954). Wenn die Ergebnisse von MATIC (1956) dafür sprechen, daß das Cutin aus veresterten Polyoxyfettsäuren aufgebaut ist, so könnte nach den letztgenannten Autoren auch eine Vernetzung über Sauerstoffbrücken (Peroxydbrücken?) als Aufbauprinzip des Cutins angenommen werden (vgl. KAUFMANN 1958). Zu bedenken ist dabei jedoch, daß die zur Bildung der Primärperoxyde notwendige Lipoxydase keineswegs in allen Pflanzen vorkommt (SÜLLMANN 1943, HOLMAN 1948, FRANKE 1951), FRANKE und FREHSE 1957). Schließlich ist noch denkbar, daß beide Bindungsprinzipien — Esterbindung und Sauerstoffbrücken — nebeneinander bestehen. So unsicher der Verlauf der Cutinbildung aufgrund der geringen Kenntnisse über die chemische Konstitution des Cutins auch heute noch ist, so kann doch als experimentell erwiesen nicht nur die Ausbildung der Cutinschicht bei der Neubildung von Blättern und Früchten (HÄRTEL 1950, HUELIN und GALLOP 1951, DAVENPORT 1956, FREYTAG 1957) und deren Beeinflussung durch äußere Faktoren, wie z.B. Chemikalien (JUNIPER und BRADLEY 1958, JUNIPER 1959), sondern auch die Fähigkeit zur Neubildung verletzter oder zerstörter Teile der Schicht und zwar durch Regeneration von unten her (FRITZ 1935, GRETSCHUSCHNIKOW und JAKOWLEWA 1951, SUCHORUKOW 1958) angesehen werden.

Abbau des Cutins

Über einen möglichen enzymatischen Abbau des Cutins liegen bisher keine experimentellen Befunde vor, obwohl eine große Anzahl phytopathologischer Arbeiten (vgl. GÄUMANN 1951) in diese Richtung weisen. Das beobachtete Eindringen phytopathogener Keime in Blätter oder Früchte wurde in enzymatischer Hinsicht bisher im Hinblick auf die Aktivität cellulolytischer und pectolytischer Enzyme (vgl. Übersichtsreferat von KERTESZ 1951) untersucht, die sich auch in einer großen Anzahl von Pilzen, insbesondere in *Aspergillus-*, *Penicillium-*, *Cladosporium-* und *Pullularia*-Arten, sowie auch in vielen Bakterien nachweisen ließen (WIERINGA 1955, SCHAEFER 1957, ALEXEJEW 1956, WOOD 1956, SINGH und WOOD 1956, WOOD und GUPTA 1958, HUSAIN und RICH 1958, HUSAIN und KELMAN 1958, KOHLMEYER 1956, 1958, ECHAUDI, van GUNDY und WALKER 1957, SKOSS 1955, GOTO und OKABE 1958). Die Frage, wie der Erreger die Cutinmembran durchdringt, wird entweder nicht diskutiert, oder aber es wird angenommen, daß zwar Cellulose und Pectin enzymatisch aufgelöst werden, das Cutin jedoch aufgrund der Befunde älterer Arbeiten (MYOSHI 1895) mechanisch durchbohrt wird (GÄUMANN 1951, SCHWEIZER 1958), wozu jedoch Drücke von etwa 7 Atm. notwendig wären. Immerhin weisen einige Beobachtungen auf enzymatische Vorgänge während der Infektion hin. So beobachteten YARWOOD (1957), PRISTOU und GALLEGLY (1954) und PURDY (1958) die Bildung einer deutlichen Einbuchtung unterhalb des Appressoriums, die durch Auflösung der Cutinschicht entstanden sein kann, da es sich nicht um ein Durchbiegen der gesamten Kutikularschicht handelt. SCHWEIZER (1958) stellte eine Schleimbildung am Appressorium fest und kreisförmige Perforationsstellen nach dem Eindringen des Erregers. Während FULTON (1948) ein An-

schwellen der Infektionshyphe innerhalb der Cutinschicht beschrieb, sprach MILLER (1949) erstmals die Vermutung aus, daß bei der Infektion von Olivenblättern, deren Kutikula zwei Cutinlagen untereinander enthält, der zwischen beiden Lagen wachsende Pilz (*Cycloconium oleaginum*) über cutin-spaltende Enzyme verfügen muß. Die Annahme einer enzymatischen Cutinspaltung wird weiterhin dadurch gestützt, daß auch Organismen, die keine Appressorienbildung zeigen, zur Durchdringung der Kutikula mit anschließender Infektion der Wirtspflanze befähigt sind, wie es neuerdings von KILPATRICK (1959) bei einer Hefe (*Rhodotorula glutinis*) mit verschiedenen Kleesorten, Bohnen und Erbsen als Wirt, beschrieven worden ist.

Außer in Bezug auf phytopathologische Fragen ist das Problem der Kutikula-Durchdringung auch in Hinsicht auf die eingangs erwähnte Selbststerilität bestimmter Pflanzen von Bedeutung (LINSKENS 1955, 1959, CHRIST 1959). Zwar konnten in den Pollen bzw. Pollenschläuchen bisher keine cutin-lösenden Enzyme nachgewiesen werden (CHRIST 1959), doch wurden an den Stellen wo der Pollenschlauch in den Stempel eingedrungen war, kreisrunde Löcher mit glattem Rand festgestellt, was für eine enzymatische Auflösung spricht, da beim Eindringen durch Druck Öffnungen mit ausgefransten Rändern zu erwarten wären (CHRIST 1959).

Neuere Arbeiten (FORSYTH, HAYWARD und ROBERTS 1958, HAYWARD, FORSYTH und ROBERTS 1959) über eine in Form kleiner Granula (LEMOIGNE, DELAPORTE und CROSON 1944, CARR 1958) in verschiedenen Bakterien vorkommenden wachsähnliche Substanz, die wahrscheinlich als Reservestoff dient (MACRAE und WILKINSON 1958) und dem Cutin rein chemisch recht nahe verwandt ist, da sie nach WILLIAMSON und WILKINSON (1958) hauptsächlich aus Poly-ß-hydroxybuttersäure besteht, zeigten, daß Synthese und Abbau der Verbindung von den Kulturbedingungen abhängig ist, da in Abhängigkeit hiervon unterschiedliche Mengen in den Bakterien gebildet wurden. Daraus ergibt sich, daß die enzymatische Bildung und Auflösung derartiger Fettsäurepolymerer grundsätzlich im physiologischen Bereich möglich sind.

Schließlich ergibt auch die völlige Destruktion des Ausgangsmaterials bei der Bildung des Waldstreus (WIERINGA 1955), der Moderfäule des Holzes (KOHLMEYER 1958), der Verrottung von Gartenabfällen bei der Kompostierung (STÖCKLI 1954, GERRETSEN 1957, FARKASDI 1958, HEINEN 1960), der Mull- und Humusbildung (MEYER 1959) und der Naßfäule der Pflanzen (ECHAUDI, van GUNDY und WALKER 1957) einen deutlichen Hinweis auf die Existenz derartiger Enzyme, die zum Abbau hochpolymerer Fettsäureverbindungen wie Cutin befähigt sind.

FRAGESTELLUNG

Daß das Problem des enzymatischen Cutin-Abbaus bisher nicht bearbeitet worden ist, ist wohl zur Hauptsache auf die damit verbundenen methodischen Schwierigkeiten zurückzuführen: Cutin ist sowohl in Wasser als auch in den üblichen organischen Solventien unlöslich, und läßt sich nur durch Hydrolyse in alkoholischem Alkali auflösen, wobei aus dem Polymerisat die freien Fettsäuren (bzw. ihre Seifen) entstehen, so daß damit die Untersuchung der Primärreaktion, eben der enzymatischen Hydrolyse, ausgeschlossen ist. Enzymatische Untersuchungen an unlöslichen Substraten wurden bisher nicht sehr häufig durchgeführt. Immerhin ist die Kultivierung cellulosespaltender Pilze auf Cellulosenährböden in Form von Filtrierpapier, Watte, oder amorphen Fällungen verschiedentlich ausgeführt worden (FAHRAEUS 1947, REESE, GILLIGAN und NORKRANS 1952, SIU und REESE 1953, SIU 1954, KOHLMEYER 1956, SCHAEFER 1957). Die Reaktion wurde dabei durch Gewichtsabnahme des unlöslichen Substrates oder durch den Nachweis der Spaltprodukte verfolgt. Ähnliche Methoden wurde auch bei der Verfolgung des komplizierteren Ligninabbaus (BIRKINSHAW und CHAPLEN 1955, BIRKINSHAW, CHAPLEN und FINDLAY 1957, COOKE 1957) angewendet.

In der vorliegenden Arbeit wird versucht, den enzymatischen Abbau des Cutins direkt und indirekt nachzuweisen, um somit Aufschluß über den Ablauf der Reaktionen zu erhalten.

Methodik

1. *Pilzmaterial und Züchtung*

Zu den geplanten Untersuchungen kamen in erster Linie solche Organismen in Frage, bei denen die Existenz cellulolytischer oder pectolytischer Enzyme als gegeben angenommen werden konnte, also phytopathogene oder holzzerstörende Pilze. Drei der hier verwendeten Stämme stammten aus der Institutssammlung, nämlich *Fusicladium cerasi* (*Rabh.*) *Sacc.* (= Venturia cerasi AD.), der Erreger des Kirschen- und Pfirsichschorfs (Schweizer 1958), *Fusarium moniliforme*, der als Holzzerstörer bekannt ist (Kohlmeyer 1956) und *Rhodotorula glutinis Var. rubescens*, eine Hefe welche nach Kilpatrick (1959) bei Klee, Bohnen und Erbsen chlorotische Verfärbung der Blätter und Beschädigungen der Samen hervorruft. Weitaus die meisten Versuche wurden jedoch mit einem *Penicillium spec.* durchgeführt, das von einem verrottenden Blatt isoliert wurde. Die Stammkulturen wurden auf Malz- oder Hafer-flocken-Agar gehalten.

Zum direkten Nachweis des Cutinabbaus wurden die Pilze auf Malzextrakt oder Czapek-Dox-Lösung in kleinen Petrischalen angezüchtet bis sich ein feines Mycel gebildet hatte. Sodann wurde diese Nährlösung vorsichtig abgegossen und nach mehrmaligem Unterspülen mit Wasser eine Nährlösung, bestehend aus 0,5 % Ammonsulfat, 0,5 % prim. K-Phosphat, 0,1 % Calciumnitrat, 0,1 % Magnesiumsulfat und einer Spur Eisenchlorid, zugesetzt. Als einzige C-Quelle wurden 0,25 % Cutin zugegeben. In verschiedenen Fällen wurde das Cutin in Form kleiner Scheibchen von ca. 1,5 cm^2 Größe zugesetzt. Auf diesen Lösungen entwickelten sich die Pilze nur wenig weiter und sporolierten sehr rasch.

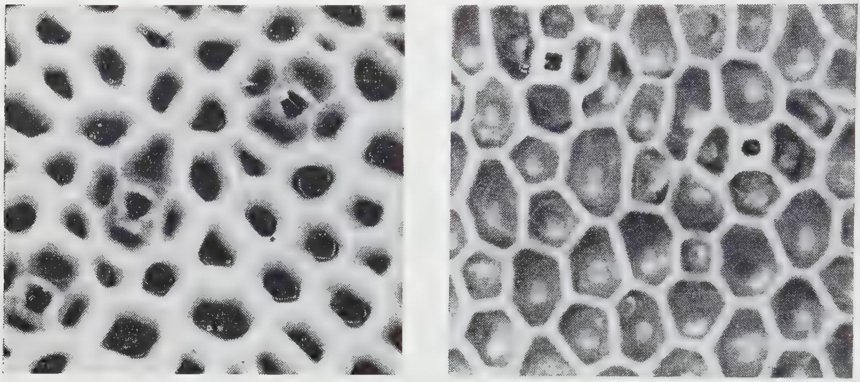
Da zu den enzymatischen Versuchen größere Mycelmengen notwendig waren, wurden die Pilze dazu nicht auf cutin-, sondern kohlenhydrathaltigen Nährlösungen angezüchtet. Als Nährlösung diente dabei bei *Penicillium spec.* Czapek-Dox-Lösung, bei *Fusarium moniliforme* eine Nährlösung nach Gaertner (1958) mit Kartoffeldekokt als C-Quelle, bei *Fusicladium cerasi* Malzextrakt; *Rhodotorula glutinis* wurde auf Malz-Agar in Kolleschalen gezüchtet. Die Petrischalen bzw. Kolleschalen wurden jeweils 3–4 Tage bei 28° gehalten.

2. *Präparate*

Von den verwendeten Substraten stammten Myristin-, Palmitin- und Stearinsäure aus Institutsbeständen, Öl-, Linol- und Linolensäure von Schuchardt (München). Die substituierten Fettsäuren α-Oxystearinsäure, 12-Oxystearinsäure und 9, 10-Dioxystearinsäure waren Präparate aus der enzymchemischen Abteilung des Instituts für Gärungswissenschaft und Enzymchemie der Universität Köln, die uns von Herrn Professor Dr. W. Franke freundlicherweise zur Verfügung gestellt wurden.

Freie Cutin-Fettsäuren (Cutin-Hydrolysat) wurden nach Roelofsen (1952) und Matic (1956) durch mehrstündiges Kochen des fein gemahlenen Cutins mit alkoholischem Alkali am Rückfluß gewonnen. Als Pflanzenmaterial zur Gewinnung von Cutin diente *Gasteria verricuosa*, die sich durch eine besonders dicke Kutikula auszeichnet. Die Trennung der Cutinschicht von den darunterliegenden Pectin- und Celluloseschichten geschah nach der Methode von Orgell (1955) durch mehrstündiges Schütteln von ca. 1,0–1,5 cm^2 großen Blattscheibchen in einer 3 %igen Pectinaselösung in 0,1 n Acetatpuffer von pH 4,0. Bei den glatten Stückchen aus dem Mittelteil der Blätter erfolgte die Ablösung der Cutinschicht bereits nach 12 Stdn.; nach 36 Stdn. war die Trennung bei allen Stückchen vollständig. Die weitere Behandlung erfolgte nach der Methode von Legg und Wheeler (1925) durch 4–5–maliges 24-stündiges Extrahieren mit absol. Alkohol am Rückflußkühler zur Entfernung der Wachse und eventuell vorhandener freier Fettsäuren. Die Entfernung der Cellulose geschah durch 5–6-malige Behandlung des Materials mit Kupferammoniak, wobei zu bemerken ist, daß die Ablösung der Cutinschicht

durch die Pectinase so sauber vor sich geht, daß nur in den Blattecken, in denen offenbar eine direkte Verbindung der Celluloseschicht mit der Cutinschicht ohne Pectinzwischenlage punktweise gegeben ist, noch geringe Reste von Cellulose vorhanden sind. Der Fortgang der Cutinisolierung wurde außer mit den üblichen Färbungsmethoden durch Röntgenaufnahmen von den einzelnen Schritten verfolgt. In Abb. 1 zeigt Bild 1 das Aussehen des Materials nach der Behandlung der Blatt-



1 2

Abb. 1. Röntgenmikroskopische Verfolgung der Vorgänge bei der Cutin-Isolierung von Blättern von *Gasteria verricuosa*.
1 – nach Behandlung der Blattstückchen mit Pectinaselösung
2 – nach Entfernung der Wachsschicht durch Extraktion in absol. Alkohol.

scheibchen mit der Pectinaselösung; in Bild 2 ist das gleiche Stück nach Entfernung der Wachsschicht durch die Alkoholbehandlung wiedergegeben: das Verschwinden des Wachsbelages ist deutlich zu erkennen. Aufnahmen nach der Kupferammoniakbehandlung zeigten, daß keine weiteren Veränderungen mehr festzustellen sind.

Die getrockneten Scheibchen wurden sodann in einer Schlagmühle zu feinem Pulver gemahlen und anschließend die Behandlung mit absol. Alkohol nochmals 12 Stdn. wiederholt, um eventuell freigesetzte Fettsäuren aus dem Innern der Cutinschicht (Hilkenbäumer 1958) zu entfernen. Zum Schluß wird das Cutin mehrmals mit HCl und heißem Wasser gewaschen und anschließend getrocknet. Man erhält ein braunes, elastisches Material das trocken aufbewahrt wird.

3. *Herstellung der Enzymlösungen*

Das in einer Öldruckpresse trocken gepreßte, zuvor mehrfach gewässerte Pilzmycel wurde mit der gleichen Menge Seesand und der 1,5–2,0-fachen Menge Wasser oder m/5 Phosphatpuffer von pH 7,6 verrieben, sodann etwa 30 Min. zur Extraktion stehen gelassen (im Kühlschrank bei + 5°) und anschließend 12 Min. bei 2000 g zentrifugiert. In vielen Fällen wurde das Mycel zunächst wie angegeben mit Wasser extrahiert und nach dem Zentrifugieren der Rückstand nochmals mit Phosphatpuffer extrahiert. Die so erhaltenen meist schwach getrübten Extrakte wurden in Eiswasser aufbewahrt. Trockengewichtsbestimmungen erfolgten bei 110° nach 48-stündiger Dialyse gegen fließendes Leitungswasser.

In verschiedenen Fällen wurden die Enzymlösungen vor ihrer Verwendung im Cellophanschlauch (2,2 cm ø) 2–4 Stdn. gegen dest. Wasser bei + 5° dialysiert.

4. *Reaktionsmessungen*

a. Zur direkten Verfolgung der cutinabbauenden Wirkung von Pilzen wurde das normal angezüchtete Mycel mit der oben (Abschn. 1) angegeben Nährlösung unterschichtet und mehrere Cutinstückchen als C-Quelle zugesetzt. Nach verschieden langer Einwirkungszeit des Pilzes auf die Scheibchen wurden diese aus der Lösung entnommen, der darauf haftende Pilz vorsichtig abgehoben und sodann

in Wasser gespült. Nach dem Trocknen wurden die Stückchen mikroskopisch untersucht und z.T röntgenmikroskopische Aufnahmen gemacht, oder von der Oberfläche Abdrücke mit Technovit hergestellt (SCHREIL 1955) und davon Aufnahmen gemacht.

b. Die Dehydrase-Wirkung wurde nach der Thunberg-Methodik bei 30° mit 2.6-Dichlorphenol-indophenol als H-Acceptor untersucht. Der "Normalansatz" hatte folgende Zusammensetzung:

0,8 ml Enzymlösung	0,9 ml H_2O
1,0 „ m/10 Phosphatpuffer pH 6,0	3,0 mg Cutin
0,3 „ m/500 Farbstofflösung	

Veränderungen dieses Ansatzes sind im Text jeweils vermerkt. Neben den Entfärbungszeiten (in Minuten) des vollständigen Ansatzes (t) und des substratfreien (t_0) wird im folgenden die Dehydrierungsintensität

$$I = 100(^1/t - {}^1/t_0)$$

und die spezifische Dehydrierungsintensität

$$I_0 = I/mg \text{ Enzymtrockengewicht}$$

angegeben. Bisweilen wird unter der Rubrik "% I_0" die prozentische Aktivität, bezogen auf I_0 des Ausgangsextraktes, angegeben. Die Bestimmung des Endpunktes der Entfärbungsreaktion geschah entweder optisch, oder aber Reaktionsgeschwindigkeit und -Endpunkt wurden photometrisch bestimmt.

c. Die Aktivitätsprüfungen nach der Warburg-Methodik erfolgten bei 30° unter Luft mit dem "Normalansatz" gleicher Zusammensetzung wie bei den anaeroben Versuchen. Als Maß der Aktivität diente die aus der Anfangsgeschwindigkeit (10 Min.) errechnete Atmungsgröße

$$Q_{O_2} = \frac{\text{m}^3\text{m } O_2}{\text{mg Enzymtrockengew.} \times \text{Stdn.}}$$

Veränderungen des Ansatzes in Bezug auf die Enzym- oder Substratkonzentration oder Variation des pH-Wertes sind jeweils im Text angegeben.

VERSUCHSERGEBNISSE

A. ORIENTIERENDE VERSUCHE

Die einleitenden Versuche hatten den Zweck, festzustellen, ob bei dem von rottenden Blättern isolierten *Penicillium spec.* eine Aktivität gegenüber Cutin überhaupt vorhanden war. Dazu wurde dem normal angezüchteten Pilz eine anorganische Nährlösung mit fein gemahlenem Cutin als einziger C-Quelle als Nährsubstrat angeboten und das weitere Wachstum verfolgt. Es zeigte sich dann (Fig. 1), daß sich das Mycel nur sehr schwach weiterentwickelte und früher als normal sporolierte.

Um mögliche Veränderungen am Cutin während der Verwendung als Substrat beobachten zu können, wurde es in der folgenden Versuchsreihe nicht in gemahlenem Zustand, sondern in Form kleiner Scheibchen von etwa 1,5 cm² Größe zugesetzt. In diesem Fall war zu beobachten, daß sich das Mycel an die Stükchen lose anheftet und darauf — wenn auch recht schwach, wie beim vorherigen Versuch und unter frühzeitiger Sporolation — weiterwuchs. Anhand von Röntgenaufnahmen und noch besser an den davon erhaltenen Umkehrfotos ließ sich die Veränderung des Materials während der Einwirkung der Pilzenzyme gut verfolgen. In Abb. II sind die Abbau-

stadien, mit der Einwirkungszeit 0 Tage = Kontrolle (1), 14 Tage (2), 21 Tage (3) und 28 Tage (4) wiedergegeben. Die Verdickungen der Cutinschicht, die zuvor die Epidermiszellen begrenzten, wie es aus dem Querschnitt der Abb. III, 1 zu ersehen ist und die hier als "Zell-

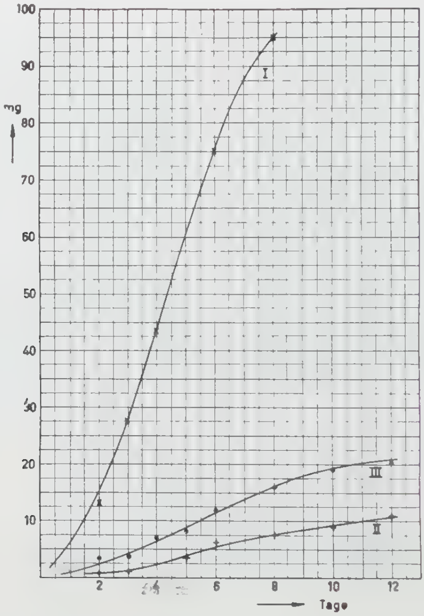


Fig. 1. Wachstumskurven von *Penicillium sp.* auf vollsynthetischer Nährlösung mit Glucose (I), reinem Cutin (II) und unvollständig gereinigtem Cutin, mit Spuren von Wachs und Celluloseresten (III) als jeweils einziger C-Quelle.

wände" erscheinen, machen zunächst einen starren und festen Eindruck. Nach zweiwöchiger Einwirkung der Pilzenzyme erscheinen sie dagegen aufgequollen und außerdem sind dünnere, "angenagte" Stellen zu erkennen. Nach drei Wochen ist besonders in den Eckpunkten eine Auflösung zu sehen und Bruchstellen in den "Wänden" sind festzustellen (s. Pfeile!). Nach vier Wochen macht sich eine deutliche Abnahme der Wanddicke bemerkbar und auch die Wandhöhe hat abgenommen, was daraus zu ersehen ist, daß hier — stärker noch als bei Bild 3 — die eigentliche "Fläche" der Cutinschicht in der gleichen Ebene erkennbar wird, während in den beiden vorausgehenden Fotos nur die "Wände" allein zu sehen sind.

Die Dickenabnahme ist auch auf den Querschnitten der Abb. III zu erkennen: Die klar begrenzten Flächen und Zähne ("Wände") der Kontrolle (1) sind nach 6-wöchiger Einwirkung des Pilzes (2) dünn und weich geworden. Diese Veränderung des Materials ist auch äußerlich erkennbar: die zunächst spröden Scheibchen werden im Laufe des fortschreitenden Angriffs weich, unelastisch und auf den Oberflächen schleimig.

Zur Verfolgung der Strukturänderungen der Oberflächen wurden

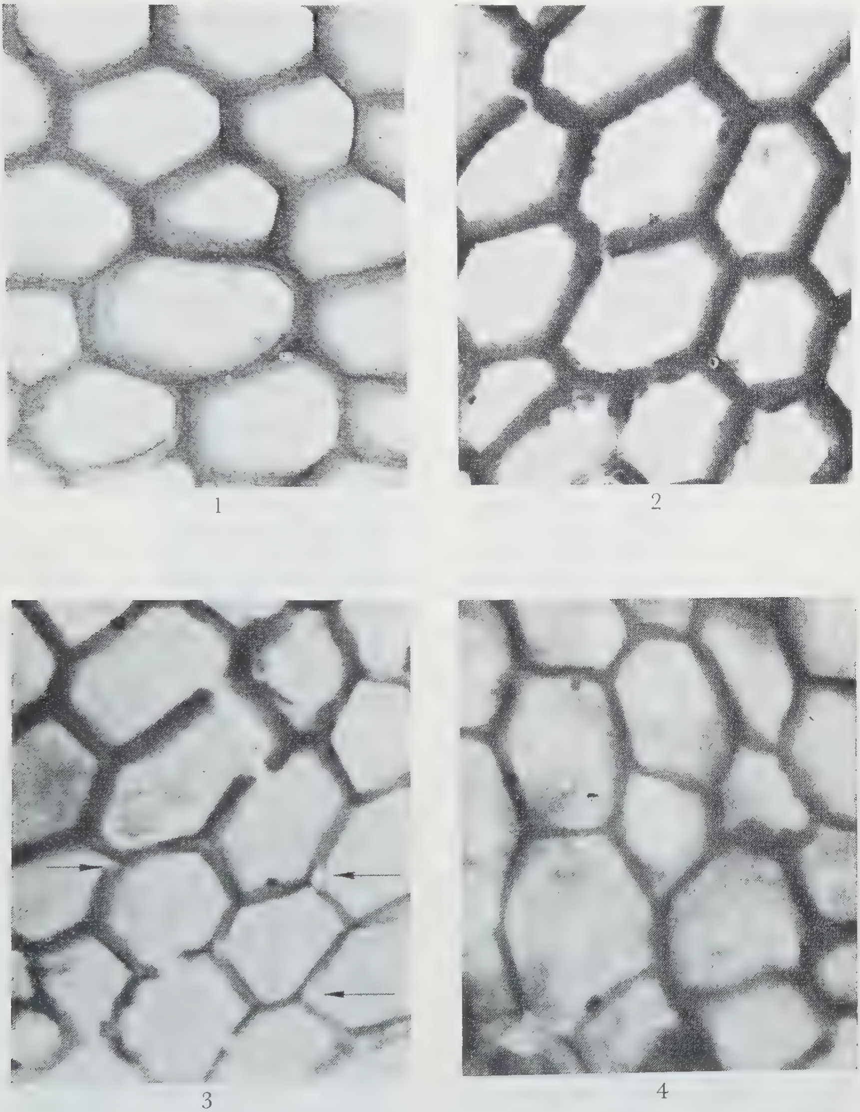
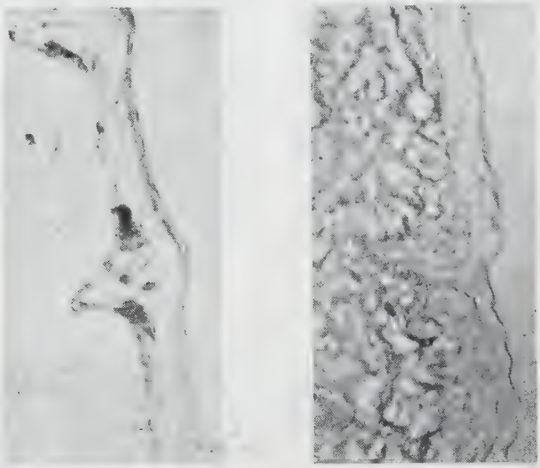


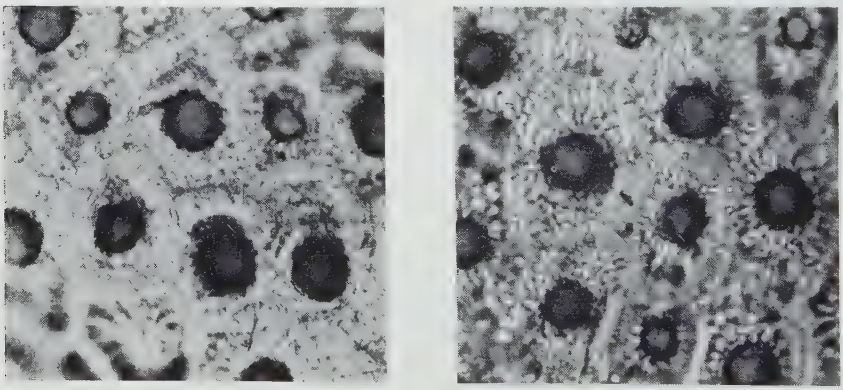
Abb. II. Veränderungen des Cutins unter Einwirkung der Enzyme von *Penicillium spec.* (Umkehrfotos von Röntgenaufnahmen) 1 = Kontrolle (Einwirkungszeit 0 Tage); 2 = Einwirkungszeit 14 Tage; 3 = Einwirkungszeit 21 Tage; 4 = Einwirkungszeit 28 Tage.

sowohl von der Außen- als auch von der Innenseite der Cutinscheibchen zu verschiedenen Zeiten Technovit-Abdrücke genommen und die Veränderungen mikroskopisch festgestellt. Abb. IV zeigt, wie sich die Oberflächenstruktur der Außenseite eines Cutinstückchens (1) bereits nach 14 Tagen (2) verändert hat. Deutlicher noch werden die Strukturänderungen auf den Aufnahmen der Innenseite der Cutinscheibchen (Abb. V, 1–4). Die beiden ersten Fotos zeigen die Struktur der Innenseite vor der Behandlung; in Bild 1 ist auf die Spitzen der Zähne scharf eingestellt, die eigentliche Fläche erscheint (wie bei den



1 2

Abb. III. Querschnitt durch ein Stück isoliertes Cutin von *Gasteria verricuosa*, 1 = Kontrolle; 2 = nach 6-wöchiger Einwirkung von *Penicillium spec.* auf das Cutin.



1 2

Abb. IV. Oberflächenstruktur der Außenseite eines Cutinscheibchens von *Gasteria verricuosa* (Technovit-Abdruck). 1 = Kontrolle; 2 = Veränderiing der Oberfläche nach 14-tägiger Einwirkung von *Penicillium spec.*

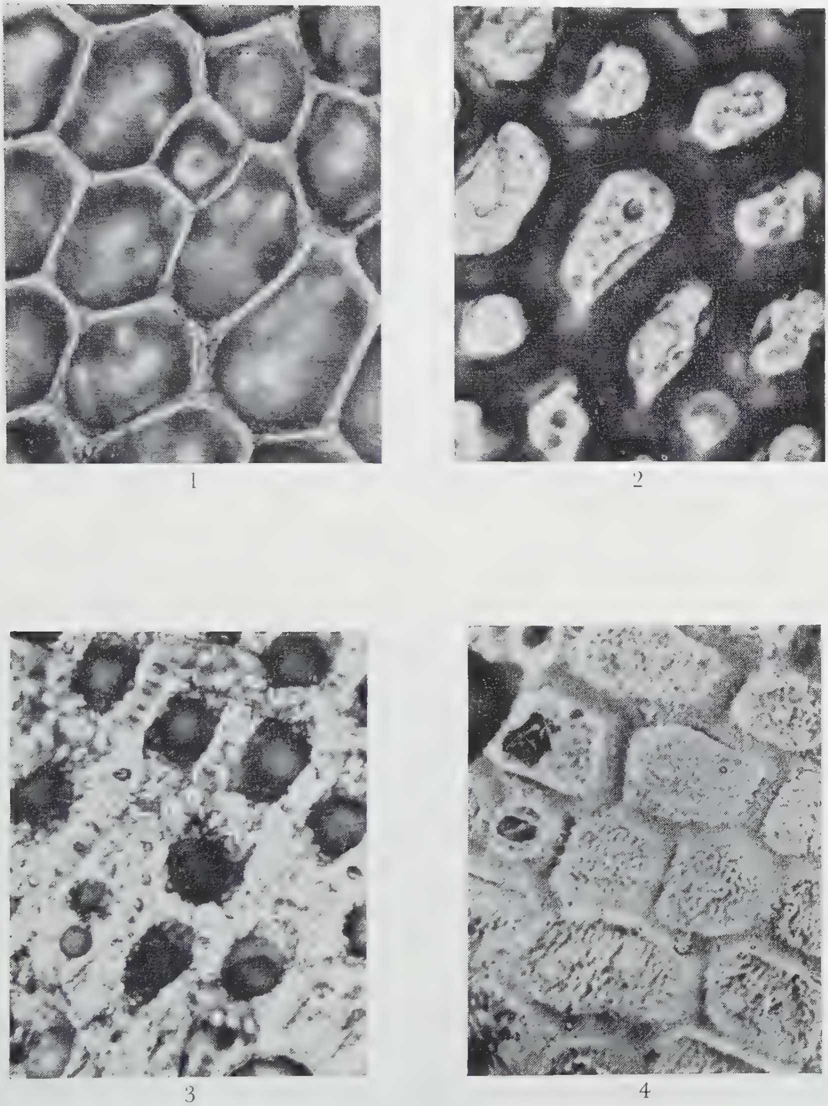


Abb. v. Veränderungen der Oberflächenstruktur der Innenseite eines Cutinscheibchens von *Gasteria verricuosa* durch Einwirkung der Enzyme aus *Penicillium spec.* 1 = unbehandeltes Stück (Kontrolle), auf die Spitzen der Zähne scharf gestellt; 2 = das gleiche Stück, auf die Flächen scharf gestellt (vgl. Text!). 3 = Struktur nach 3-wöchiger Einwirkung des Pilzes; 4 = nach 5-wöchiger Einwirkung.

Röntgenfotos) als Hohlraum. Auf grund der größeren Höhe der Zähne erscheinen diese Hohlräume von der Unterseite aus gesehen bedeutend größer als von der Außenseite her, was beim Vergleich mit der Abb. IV gut zu erkennen ist. Im folgenden Foto (2) ist auf die Fläche scharf gestellt, wodurch nun die Zähne ("Wände") breit und unscharf erscheinen. Bild 3 zeigt die Struktur nach dreiwöchiger Einwirkung des Pilzes. Hier ist wiederum auf die höchste Stelle der Zähne, deren Spitzen bereits verschwunden sind, und die deshalb breiter erscheinen, eingestellt; die "Hohlräume" sind wegen des geringeren Abstandes bereits viel kleiner. Nach fünfwöchiger Einwirkung (4) ist die Destruktion bereits so weit fortgeschritten, daß Zähne und Flächen in einer Ebene erscheinen; an einigen Eckpunkten sind beginnende, und oben rechts eine bereits vollständige Bruchstelle (wie auf den Röntgenaufnahmen der Abb. II, 3 und 4) zu erkennen.

Es scheint von Interesse, daß die vorstehenden Teile der Scheibchen, die Zähne, im Laufe der Destruktion offenbar eher angegriffen werden als die Fläche selbst und dies obwohl die Stückchen während der Einwirkungszeit stets mit der Außenseite nach oben in der Nährlösung lagen, so daß der darauf wachsende Pilz mit der Innenseite keinen direkten Kontakt hatte.

B. Enzymatische Versuche

Die weiteren Versuche liefen darauf hinaus, den stattfindenden Abbau mit enzymatischen Methoden zu verfolgen (vgl. Heinen und Linskens 1960). Ausgehend von der Überlegung, daß beim Cutinabbau nach der Aufspaltung des Polymerysates freie Fettsäuren erscheinen müssen, die (wie die orientierenden Versuche zeigten) vom Pilz als C-Quelle benutzt werden können, war in irgend einer Phase des weiteren Abbaus eine Dehydrierung dieser Fettsäuren zu erwarten, zumal Fettsäuredehydrasen in Schimmelpilzen schon verschiedentlich nachgewiesen worden sind (Mukherjee 1951, 1952, Franke und Heinen 1958). Da eine derartige Dehydrierung eine vorhergehende enzymatische Spaltung des Cutins voraussetzt, wäre damit eine indirekte Verfolgung der Spaltungsreaktion möglich.

I. Nachweis einer Dehydrase

Zunächst was also festzustellen, ob die aus dem Cutin durch Behandlung mit alkoholischem Alkali entstehenden Fettsäuren für den Pilz angreifbar waren. Dazu wurde das Hydrolysat in Thunberg-Ansätzen als Substrat einer *Mycelsupension* von *Penicillium spec.* verwendet. In ersten Versuchen zeigte sich, daß eine Dehydrierung des Hydrolasytes nachweisbar ist; zum Vergleich lief ein Ansatz mit Cutin als Substrat mit und ergab, daß dieses nur in sehr geringem Maße dehydriert wird:

Leer–Ansatz:	$t_0 = 18$ Min.	*I-Wert*
8 mg Hydrolysat:	$t_H = 12$,,	2,77
8 mg Cutin:	$t_C = 16$,,	0,69

Aus der Beobachtung, daß auch das Cutin (wenn auch in geringem Maße) durch die Mycelsuspension dehydriert wird ist zu schliessen, daß entweder freie Fettsäuren darin vorhanden sind (HILKENBÄUMER 1958), oder daß die Suspension neben der Dehydrase ein Enzym enthält, daß aus dem Cutin Fettsäuren freisetzt, worauf später eingegangen wird.

Nachdem Vorversuche ergeben hatten, daß das Enzym relativ leicht aus dem Mycel zu extrahieren ist, ergaben *Extraktionsversuche* bei verschiedenen pH-Werten (Extraktion bei pH 5.2, 5.6, 6.0, 6.8 und 7.2) einen recht weiten optimalen Extraktionsbereich von pH 5,5–6,8, so daß statt des Puffers ebenso gut Wasser zur Extraktion benutzt werden konnte. Bei der Bestimmung der pH-Aktivität der Dehydrase ergab sich ein ausgeprägtes Optimum bei pH 6,2. Die Bestimmung der optimalen Enzymkonzentration ergab einen relativ engen Bereich von

TABELLE I

Abnahme der Dehydrase-Aktivität gegenüber Cutinhydrolysat-Fettsäuren durch Alterung bei 5 und 24°) und Dialyse bei + 5°.

Enzymlösung	t_0	t	I	I_6	% I_0
frisch	34	12	5,38	0,82	100
4 Stdn. bei 24°	38	17	3,25	0,49	60
4 ,, ,, 5°	35	14	4,28	0,63	70
4 ,, Dialyse	∞	160	0,62	0,09	11,5

2,1–8,3 mg Enzymtrockengewicht pro Ansatz. Gegenüber Alterung und Dialyse zeigte sich die Dehydrase sehr empfindlich. Aus Tab. 1 ist zu ersehen, daß die Aktivität schon bei der Alterung rapide abnimmt und bei der Dialyse nach 4 Stdn. nur noch 11,5 % der Ausgangsaktivität erhalten sind. Die Ergebnisse dieser Versuche weisen darauf hin, daß die hier vorliegende Dehydrase mit der von FRANKE und HEINEN (1958) in *Aspergillus niger* und *Rhizopus tonkinensis* nachgewiesenen Fettsäure-Dehydrase wenn nicht identisch so doch zumindest nahe verwandt ist.

II. Indirekter Nachweis eines cutinspaltenden Enzyms

1. *Nachweis mittels der Thunberg-Methodik*

Bei den Versuchen zur Extraktion der Dehydrase mit Pufferlösungen bei verschiedenen pH-Werten lief neben dem Leer- und Substrat-(=Hydrolysat-)-Ansatz auch stets ein Ansatz mit Cutin als Substrat mit, da sich ja in der Mycelsuspension eine geringe Aktivität auch gegenüber dem nichthydrolysierten Cutin gezeigt hatte. Dabei ergab sich neben dem bereits genannten Aktivitätsunterschied der Dehydrase in Abhängigkeit vom Extraktions-pH-Wert auch ein Unterschied der Aktivität gegenüber dem Cutin. Wie Tab. 2 zeigt, steigt die Aktivität gegenüber dem Cutin mit steigendem pH-Wert an, wobei ein Optimum hier nicht erreicht wird. Daraus ergibt sich, daß die Aktivität der Dehydrase gegenüber Cutin, die bereits in der Mycelsuspension in geringem Maße festzustellen war, nicht auf eventuell vorhandene freie

Fettsäuren im Cutin zurückzuführen ist, sondern daß ein Enzym vorhanden sein muß, welches das Cutin aufspaltet und die Reaktionsprodukte der Dehydrase zugänglich macht.

Nachdem zuvor festgestellt worden war, daß bei Extraktion der Dehydrase mit Wasser niemals eine Aktivität gegenüber dem Cutin auftrat, wurde nun nach der wässrigen Extraktion eine solche mit

TABELLE II

Aktivität dreier bei verschiedenem Extraktions-pH gewonnener Extrakte aus *Penicillium spec.* gegenüber Cutinhydrolysat-Fettsäuren und Cutin.

Extraktions-pH	Substrat	t_0	t	I	I_0
5,8	Hydrolysat	38	13	5,13	1,02
	Cutin		20	2,37	0,48
6,5	Hydrolysat	42	15	4,28	0,85
	Cutin		17	3,50	0,69
7,3	Hydrolysat	45	35	0,66	0,13
	Cutin		16	4,03	0,80

Phosphatpuffer vom pH 6,7 durchgeführt. (Der pH-Wert wurde deshalb im genannten Bereich gewählt, weil hier Aktivität gegenüber beiden Substraten zu erwarten war, wie das Beispiel der vorhergehenden Tab. 2 zeigt.) Dabei geht die Dehydrase fast vollständig im Wasserextrakt (E_1) in Lösung; Aktivität gegenüber Cutin ist darin nicht vorhanden. Der Pufferextrakt (E_2) zeigt nur sehr geringe Aktivität gegen beide Substrate. Gibt man jedoch beide Extrakte zusammen, so zeigt sich deutlich, daß nun beide Substrate angegriffen werden, woraus sich ergibt, daß das Enzym, welches das Cutin anzugreifen vermag, durch Extraktion mit Puffer in Lösung zu bringen ist (Tab. 3).

TABELLE III

Dehydrierung von Cutinhydrolysat-Fettsäuren und unbehandeltem Cutin durch zwei verschieden gewonnene Extrakte aus *Penicillium spec.* und deren Kombination.

Enzymlösung	Trocken-Gewicht	Substrat	t_0	t	I	I_0
Wasser-Extrakt (E_1)	3,8	Hydrolysat	27	16	2,55	0,67
		Cutin		28	0	0
Puffer-Extrakt (E_2)	4,7	Hydrolysat	56	45	0,44	0,094
		Cutin		49	0,26	0,056
Kombination ($E_1 + E_2$)	8,5	Hydrolysat	38	11	6,48	0,76
		Cutin		12	5,70	0,67

Das Enzym, das die Aufspaltung des Cutins bewirkt, wird im folgenden mit allem Vorbehalt als "Cutinase" bezeichnet. Dieser Begriff dient lediglich zur Vereinfachung und soll nichts darüber aussagen, ob es sich dabei um *ein* Enzym handelt oder einen Enzymkomplex; auch in Bezug auf die Natur des Enzyms soll damit nichts festgelegt werden, denn da über den chemischen Aufbau des Cutins nichts Sicheres bekannt ist, kann es sich dabei sowohl um ein Enzym handeln

welches Esterbindungen aufspaltet (sofern man unter "Cutin" Polyester von Oxyfettsäuren versteht), als auch eine Lyase welche Äther- oder Peroxydbrücken spaltet (wenn man annimmt, daß die Fettsäuren des Cutins auf diese Weise vernetzt sind).

Da es bei der schwachen Aktivität wenig aussichtsreich erschien, den Ablauf der Reaktion durch Bestimmung der End- oder Zwischenprodukte zu verfolgen, wurde im folgenden die Dehydrierungsreaktion als Maß zur Bestimmung der Aktivität der Cutinase herangezogen. Die Methode ist natürlich nur begrenzt anwendbar, denn da nicht sicher ist, zu welchem Zeitpunkt die Dehydrierung im Laufe des Abbaus stattfindet, ist es z.B. möglich, daß bei bestimmten Extraktionsverfahren eine Aktivitätssteigerung der Cutinase nur deshalb nicht erkannt werden kann, weil möglicherweise ein Enzym, das nach der Spaltung durch die Cutinase diese Spaltprodukte weiter abwandelt, nicht zugleich mit erfaßt wird. Andererseits kann die Spaltungsreaktion auch nur so lange mittels der Dehydrierungsreaktion verfolgt werden, als die Aktivität der Dehydrase höher ist als die der Cutinase.

a. *Bestimmung des optimalen Extraktions-pH-Wertes*

Dazu wurde das Mycel von *Penicillium spec.* nach der Extraktion mit Wasser mit m/5 Acetat-, Phosphat-, oder Borat-Puffer von pH 5.6, 6.0, 6.8, 7.4, 7.9 und 8.3 (mit Überschneidungen in den kritischen Bereichen) extrahiert und die Aktivität der einzelnen Extrakte unter

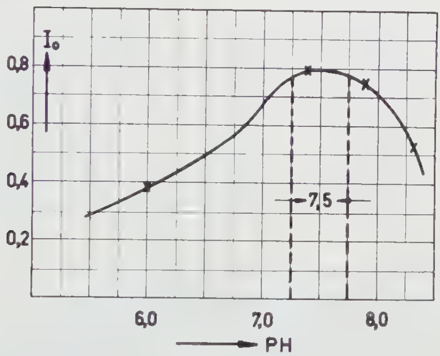


Fig. 2. Abhängigkeit der "Cutinase"-Aktivität vom Extraktions-pH-Wert. (Indirekter Nachweis durch Bestimmung der Dehydrierungsintensität des Wasser- (= Dehydrase-)—Extraktes in Thunberg-Ansätzen.

gleichzeitigem Zusatz von dehydrase-haltigem Wasserextrakt geprüft. Das Ergebnis ist in Fig. 2 wiedergegeben: Der optimale Extraktionsbereich liegt zwischen pH 7,25 und 7,75; zur alkalischen Seite hin fällt die Aktivität rascher ab als zur sauren Seite.

b. *Alterung und Dialyse*

Im Laufe der Versuche zeigte sich, daß der Pufferextrakt bedeutend stabiler war als der Wasserextrakt. Nach 48-stündiger Aufbewahrung des Pufferextraktes bei +5° war die Aktivität der Cutinase erst um 45 % gesunken. Ein mit Phosphatpuffer von pH 7,6 gewonnener Extrakt aus Penicillium spec. wurde daher 3 Stdn. bei +5° gegen dest. Wasser dialysiert und anschließend die Aktivität des Extraktes in

Kombination mit dem Dehydrase-Extrakt bestimmt (Tab. 4). Zum Vergleich wurde auch der Wasserextrakt dialysiert und dessen Einfluß auf die Aktivität untersucht. Zur Kontrolle lief ein Ansatz mit Pufferextrakt mit, der 10 Min. im siedenden Wasserbad gehalten worden war. Aufgrund dieser Ergebnisse ist die Cutinase beständig gegen Dialyse und benötigt also keine dialysablen Cofaktoren. Weitere Versuche ergaben, daß auch nach 6-stündiger Dialyse nur ein geringer Aktivitätsverlust (28 %) festzustellen ist, der wohl auf eine beginnende Schädigung des Enzymeiweiß zurückzuführen ist.

TABELLE IV

Einfluß der Dialyse auf die Aktivität des Wasserextraktes und des Pufferextraktes aus *Penicillium spec.* mit Cutin-Fettsäuren und Cutin als Substrat. (Dialyse: 3 Stdn. gegen dest. Wasster bei + 5°).

Enzymlösung	Substrat	t_0	t	I	I_0
Wasser-	Hydrolysat	27	13	4,00	0,76
Extrakt (E_1)	Cutin		29	0	0
Puffer-	Hydrolysat	69	74	0	0
Extrakt (E_2)	Cutin		65	0,09	0,02
Kombination	Hydrolysat	35	9,5	7,65	0,81
($E_1 + E_2$)	Cuttin		7	11,45	1,22
E_1 dialysiert	Hydrolysat	41	37	0,26	0,03
	Cutin		44	0	0
E_2 dialysiert	Hydrolysat	65	65	0	0
	Cutin		55	0,28	0,03
E_2 + E_1-dia-	Hydrolysat	52	50	0,08	0,01
lysiert	Cutin		47	0,20	0,02
E_1 + E_2-dia-	Hydrolysat	37	10	7,30	0,78
lysiert	Cutin		6	13,90	1,48
E_1 + E_2-(er-	Hydrolysat	23	9	6,75	0,72
hitzt auf 100°)	Cutin		23	0	0

c. *Zur Zeitabhängigkeit der Cutinase-Wirkung*

Zur Klärung der Frage, ob die Dehydrierungsintensität des Wasserextraktes von der Einwirkungsdauer des Pufferextraktes abhängig ist, wurden Leer- und Substratansätze mit dem Pufferextrakt verschieden lange im Wasserbad bei 30° gehalten, dann die Dehydraselösung zugesetzt und die Dehydrierungsintensität bestimmt. Der Vergleich mit der Dehydrierungsintensität die bei der direkten Zugabe der Dehydraselösung erreicht wird (also Einwirkung der Cutinaselösung = 0 Min.) ergab, daß die Intensität der Dehydrierung mit der Länge der Einwirkungszeit der Cutinaselösung bis zur Erreichung eines Optimums ansteigt (Fig. 3). Da die zum Vergleich mitlaufenden Ansätze mit Stearinsäure als Substrat keinen Intensitätsanstieg zeigten, deren Dehydrierung also unabhängig von der Einwirkung der Cutinase ist, ergibt sich daraus einerseits, besonders da beide Substrate in vergleichbarer Konzentration vorlagen, daß es sich bei den beobachteten Effekten nicht um die Dehydrierung eventuell im Cutin vorhandener freier Fettsäuren handelt, deren Dehydrierung von einer vorherigen Spaltung unabhängig sein müßte, und andererseits, daß der enzymatische Cutinabbau durch eine Spaltungsreaktion eingeleitet wird.

Die Tatsache, daß nach etwa 30-minütiger Einwirkung ein Optimum erreicht wird, nach dem die Dehydrierungsintensität nicht mehr ansteigt, ist wohl darauf zurückzuführen, daß zu diesem Zeitpunkt die Aktivitätsgrenze der Dehydrase erreicht ist.

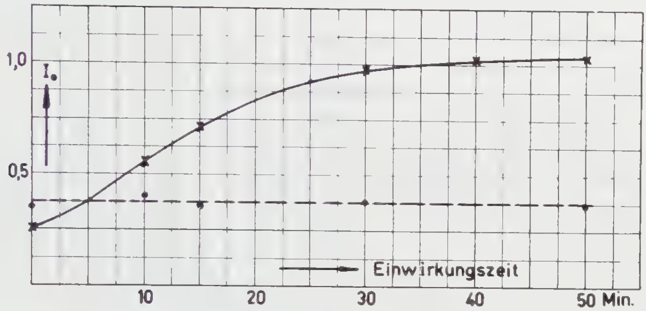


Fig. 3. Einfluß der Einwirkungsdauer der Cutinaselösung auf die Intensität der nachfolgenden Dehydrierung.
x——x = 3 mg Cutin/Ansatz
•----• = m/150 (= 5,5 mg) Stearat/Ansatz

Die recht geringe Aktivität der Dehydrase ergab sich auch aus folgenden Versuchen: Hält man die Konzentration der Dehydrase im optimalen Bereich (2,1–8,3 mg/Ansatz) konstant und setzt steigende Mengen Cutinaselösung zu, so ergibt sich eine Steigerung der Dehydrierungsintensität in einem Bereich von 1,3–5,4 mg Cutinaselösung (Enzymtrockengewicht) pro Ansatz. Bei weiterer Konzentrationserhöhung steigt die Dehydrierungsintensität nicht mehr an und bei sehr hohen Konzentrationen ist sogar ein Aktivitätsabfall zu erkennen. Das bedeutet, daß durch die Cutinase so viel Substrat für die Dehydrase freigesetzt wird, daß ihre Aktivität zur Dehydrierung desselben nicht mehr ausreicht und schließlich durch den Substratüberschuß eine Schädigung des Dehydrierungsenzyms bzw. eine Blockierung oder Hemmung eintritt.

2. *Nachweis mittels der Warburg-Methodik*

In aeroben Versuchen ergab sich, daß sowohl die durch Extraktion des Mycels von Penicillium spec. mit Wasser als auch mit Puffer von pH 7,6 erhaltenen Lösungen das Cutin zu oxydieren vermögen (Fig. 4). Der bei den anaeroben Versuchen erhaltene Befund, daß die beiden Lösungen verschiedene Empfindlichkeit gegen Dialyse besitzen, konnte in diesen Versuchen bestätigt werden: Nach 4-stündiger Dialyse sinkt die Aktivität des Pufferextraktes um etwa 26 % ab, wobei insbesondere die Anfangsgeschwindigkeit der Oxydationsreaktion verringert ist, während die Aktivität des Wasserextraktes auf etwa 10 % der Ausgangsaktivität zurückgeht.

Zur Prüfung der Frage, ob auch unter aeroben Bedingungen eine Abhängigkeit der Cutinasewirkung von der Einwirkungszeit gegeben ist, wurde zu Leer- und Substratansätzen mit Pufferextrakt nach resp. 10, 20 oder 40 Min. der Wasserextrakt aus dem Seitenarm zugekippt und der Verlauf der Oxydation verfolgt. Es ergab sich, daß die Intensität der Oxydation in Abhängigkeit von der Einwirkungszeit der Cutinase zunimmt (Fig. 5). Auch hier verläuft der Aktivitätsanstieg nicht linear, sondern nimmt mit zunehmender Dauer der Inkubationszeit langsam ab.

Da das Primärenzym der Cutinspaltung im Pufferextrakt vorliegt, wurde der Wasserextrakt zunächst nicht weiter untersucht und die folgenden Versuche am Pufferextrakt ausgeführt. Orientierende Versuche ergaben, daß die Aktivität gegenüber Cutin bei Fällung der Eiweiße mit Ammonsulfat bei 80 %iger Sättigung erhalten bleibt (Fig. 6), und daß bei fraktionierter Fällung in verschiedenen Frak-

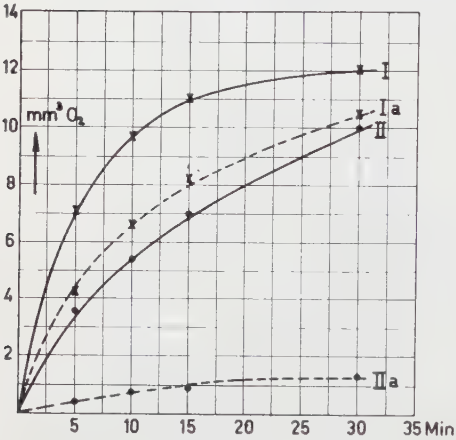


Fig. 4

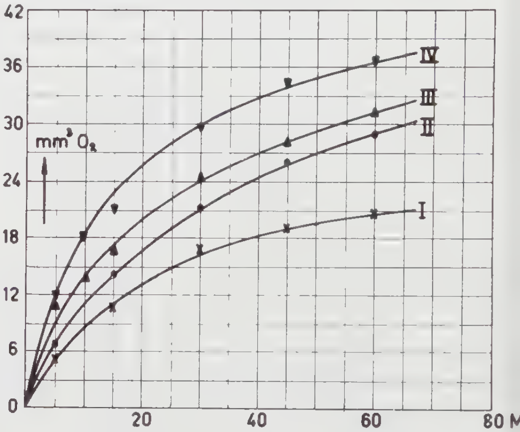


Fig. 5

Fig. 4. Cutin-Oxydation durch Pufferextrakt (I) und Wasserextrakt (II) aus *Penicillium spec.*
Abfall der Aktivität nach 4-stündiger Dialyse gegen dest. Wasser beim Pufferextrakt (Ia) und beim Wasserextrakt (IIa).

Fig. 5. Cutin-Oxydation bei Zugabe des Wasserextraktes nach O (I), 10 (II), 20 (III) oder 40 (IV) Minuten.

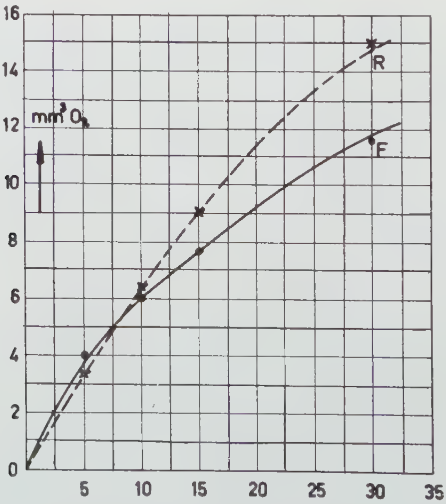


Fig. 6. Oxydation von Cutin durch den Rohextrakt aus *Penicillium spec.* (*R*) und die daraus bei 80 %iger Sättigung mit Ammonsulfat erhaltene Fällung (*F*).

tionen Aktivität nachweisbar ist. Aus den bisherigen Ergebnissen ist jedoch noch nicht mit Sicherheit zu schließen, ob mehrere Enzyme am Cutinabbau beteiligt sind und welches für den ersten Angriff des Substrates verantwortlich ist.

III. Einige Spezifitätsversuche

Da bisher nicht eindeutig geklärt werden konnte, ob im Cutin gesättigte, ungesättigte oder oxydierte Fettsäuren vorliegen (Literatur s. Einleitung), schien es sinnvoll, die Aktivität des cutinasehaltigen Extraktes gegenüber verschiedenen Substraten zu prüfen. Dazu mußte jedoch zunächst die Substratspezifität der Dehydrase bekannt sein und in Thunberg-Versuchen ergab sich, daß praktisch nur gesättigte höhere Fettsäuren angegriffen werden, aber auch Oxyfettsäuren dehydriert werden, ähnlich der von Franke und Heinen (1958) in *Aspergillus niger* und *Rhizopus tonkinensis* nachgewiesenen Schimmelpilz-Dehydrase. Von den ungesättigten Fettsäuren der C//-Reihe wird, wie Tab. 5 zeigt, nur die Ölsäure noch sehr schwach dehydriert, während Linol- und Linolensäure nicht als H-Donatoren dienen können.

TABELLE V

Dehydrierung gesättigter höherer Fettsäuren sowie Oxyfettsäuren und C_{18}-Fettsäuren mit steigender Anzahl Doppelbindungen durch die Fettsäure-Dehydrase aus *Penicillium spec.*

Substrat	t_0	t	I	I_0
Myristinsäure		11	5,25	0,94
Palmitinsäure		12	4,48	0,80
Stearinsäure		11	5,25	0,94
Ölsäure	26	17	2.03	0,36
Linolsäure		29	0	0
Linolensäure		30	0	0
α-Oxystearinsäure . . .		16	2,40	0,43
9, 10-Dioxystearinsre. .		21	0,91	0,16

Nachdem in aeroben Versuchen dann zunächst festgestellt wurde, daß alle verwendeten Substrate, nämlich Stearinsäure, α-Oxystearinsäure, 12-Oxystearinsäure, 9,10-Dioxystearinsäure und Ölsäure durch den cutinasehaltigen Extrakt angegriffen werden, wurde anschliessend in anaeroben Ansätzen die Dehydrierbarkeit der genannten Substrate in Gegenwart beider Extrakte geprüft. Wie aus Fig. 7 zu ersehen ist, werden bei Zusatz der Dehydraselösung nach 10 Min. langer Inkubation der Substrate mit der Cutinaselösung die beiden Monooxyverbindungen besser angegriffen als die unsubstituierte Stearinsäure und die übrigen Substrate. Dieser Effekt wird nach 1-stündiger Inkubation noch deutlicher: Die Aktivität gegenüber der α-Oxystearinsäure und dem Cutin erhöht sich in etwa gleichem Maße und außerdem ist die Aktivität gegenüber der 12-Oxystearinsäure stark erhöht; bei den anderen Substraten fehlt eine derartige Steigerung entweder vollständig oder liegt innerhalb der Fehlergrenze. Wenngleich hieraus nicht auf das Vorkommen derartiger Oxyfettsäuren im

Cutin geschlossen werden kann, so ist es doch wahrscheinlich, daß solche Verbindungen bevorzugte Substrate der am Cutinabbau beteiligten Enzyme darstellen und im Laufe der Abbauprozesse auftreten.

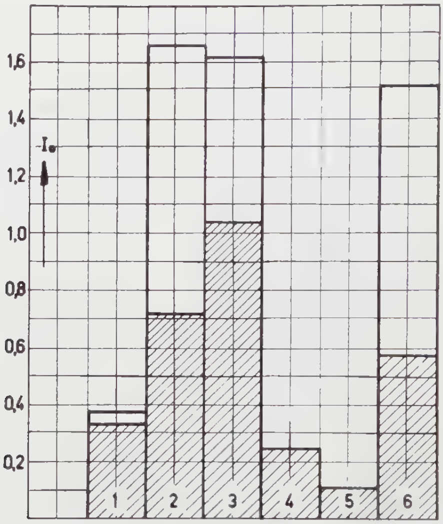


Fig. 7. Aktivität des cutinasehaltigen Extraktes aus *Penicillium spec.* gegenüber verschiedenen Oxyfettsäuren sowie Stearinsäure, Ölsäure und Cutin. (Schraffierte Stapel = Inkubationszeit 10 Min.).

1 = Stearinsäure
2 = α-Oxystearinsäure
3 = 12-Oxystearinsäure
4 = 9, 10-Dioxystearinsäure
5 = Ölsäure
6 = Cutin

IV. Zur Verbreitung cutinspaltender Enzyme

In anaeroben Versuchen wurde die Aktivität von Pufferextrakten aus *Fusarium moniliforme* und *Rhodotorula glutinis* gegenüber dem Cutin mit den bisherigen Extrakten aus *Penicillium spec.* verglichen und zwar wie in früheren Versuchen bei verschieden langen Inkubationszeiten. Wie Fig. 8 zeigt, is die Aktivität der Cutinase aus *Fusarium moniliforme* (II) verglichen mit der aus *Penicillium spec.* (I) sehr schwach; die Aktivität des *Rhodotorula*-Extraktes liegt dagegen etwa 1,7 mal höher (III). Bei Kombination des *Rhodotorula*-Extraktes mit der Dehydrase aus *Penicillium spec.* (IV) ergab sich eine weitere Aktivitätssteigerung. In weiteren Versuchen wurde eine Reihe von Laboratoriumsstämmen, darunter *Aspergillus clavatus*, *A. tamarii*, *Fusarium culmorum*, *Penicillium claviforme* und *P. notatum* auf ihren Gehalt an Cutinase untersucht, doch war bei keinem der genannten Stämme eine Aktivität nachweisbar.

V. Direkter Nachweis der Cutinspaltung

Bei der Untersuchung der Änderung der Eigenschaften des Cutins wenn es als Nährsubstrat für *Penicillium spec.* verwendet wird zeigte sich, daß sich nicht nur die mechanischen Eigenschaften ändern, wobei

aus dem harten, elastischen Material eine weiche, gummöse Masse wird, sondern auch die Löslichkeit. Cutin ist normalerweise auch in konzentriertem Alkali unlöslich und kann nur durch längeres Kochen in alkoholischem Alkali oder in absol. Alkohol mit K_2CO_3 langsam hydrolysiert werden. Das vier bis fünf Wochen als Nährsubstrat verwendete Cutin ist hingegen in warmem, verdünnten Alkali (15 %ige KOH oder NaOH) teilweise leicht löslich.

Zur genaueren Prüfung wurden je 2 mg unbehandeltes und angegriffenes Cutin 30 Min. in absol. Alkohol unter Zusatz von K_2CO_3 am Rückfluß gekocht und anschließend die Menge der in Lösung

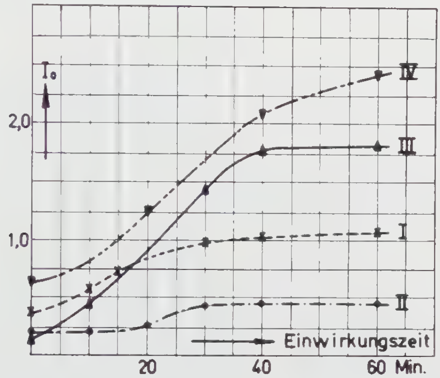


Fig. 8. Cutinase-Gehalt von Extrakten aus verschiedenen Pilzen (vgl. Text).

gegangenen Fettsäuren bestimmt. Es ergab sich, daß in der kurzen Zeit vom vorbehandelten (enzymatisch angegriffenen) Material bereits 47 % in Lösung gegangen waren, während beim Kontrollansatz nur 0,8 % des Ausgangsmaterials gelöst waren.

Aus dem Ergebnis ist zu schließen, daß durch die Einwirkung der Pilzenzyme die chemischen Eigenschaften des Cutins grundlegend verändert werden. Ob es sich dabei um eine Hydrolyse unter Aufspaltung von Esterbindungen, oder um die Lösung von Sauerstoffbrücken handelt, läßt sich hieraus allerdings nicht entscheiden. Immerhin bietet sich auf diese Weise die Möglichkeit, die recht rohe Methode der Aufspaltung des Cutins auf chemischen Wege, wobei die Bildung von Artefakten nicht ausgeschlossen werden kann, durch eine enzymatische Lyse zu ersetzen. Nach papierchromatografischer Auftrennung und Bestimmung der Spaltprodukte wird es leichter sein, Einblick in die beim enzymatischen Abbau des Cutins zu erwartenden Reaktionen zu erhalten.

SUMMARY

1. Penicillium spec., a mould isolated from rotting leaves, is able to grow on a culture medium with cutin as the only carbon source. Hence the fungus contains enzymes which are able to destroy cutin.

2. While the mould-enzymes were acting upon the cutin, the resulting variations of the nutritional substrate were followed with certain intervals as well by roentgen microscopical examinations as by replica technique; the phenomenons of breakdown could be demonstrated by means of photographs.

3. The change in mechanical properties caused by the enzymes of the fungus are also apparent macorscopically, moreover the insoluble cutin is converted into a form that is easily soluble in diluted warm alkali.

4. By means of Thunberg technique in water extracts of Penicillium spec. a dehydrogenase could be demonstrated which is able to dehydrogenate fatty acids obtained by chemical hydrolysis of cutin. Some properties of these dehydrogenase were investigated.

5. Using the dehydrogenase reaction as an assay, in extracts of Penicillium spec. with phosphate buffer the presence of an enzyme could be demonstrated that makes cutin readily attackable by the dehydrogenase by liberating free fatty acids from the polymere compound. The preliminary term "cutinase" is proposed for this enzyme.

6. Some properties of the "cutinase" were determined under aerob and anaerob conditions and the stability of the enzyme against dialysis as well as the possibility of precipitation with ammonium sulfate were established. There are indications that preferably monohydroxy stearic acids are used as a substrate by this enzyme.

7. Some results point to the fact that in buffered extracts of Penicillium spec. several enzymes are present taking part in the breakdown of cutin; probably "cutinase" consists of more than one enzyme.

8. Low "cutinase" activity could be demonstrated also in Fusarium moniliforme and higher activity in a yeast, Rhodotorula glutinis.

Herrn Professor Dr. H. F. Linskens (Nijmegen) danke ich für den Hinweis auf das Problem, anregende Diskussionen und die ständige Förderung meiner Arbeit. Ebenso danke ich Herrn Professor Dr. W. Franke (Köln) für wertvolle experimentelle Vorschläge und Literaturhinweise, sowie die freundliche Überlassung von Oxyfettsäure-Präparaten. Herrn M. M. A. Sassen danke ich für die Isolierung des Penicilliumstammes. Für experimentelle Hilfe danke ich Fräulein. I. van den Brand, für die Röntgenaufnahmen Herrn M. van den Donk und für die Ausführung der fotografischen Arbeiten Herrn H. J. M. Spruit.

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A LATE-GLACIAL LAKE DEPOSIT NEAR WASKEMEER (PROV. OF FRIESLAND)

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The numerous bowl-shaped, sometimes fairly deep depressions which may be wholly or partly filled with organic sediments constitute a characteristic feature in the ground moraine landscape of the northern Netherlands. As for the origin of these depressions, some of them were supposed to be kettle-holes (*cf.* DE WAARD 1947), while others were regarded as the product of wind erosion during the last part of the Late-glacial (VEENENBOS 1952). As it was during the Riss (Penultimate) glaciation that a part of the Netherlands was covered by the ice cap, it was difficult to understand why interglacial (Eemian) peat or gyttja is never found in the depressions considered to be kettle-holes, but only organic deposits from the Late-glacial and later. In place of the kettle-hole theory another hypothesis for the origin of this last type of depression was brought forward by MAARLEVELD and VAN DEN TOORN (1955). According to these authors, some of the depressions in the northern Netherlands are to be interpreted as the remains of pingos.

Today the formation of pingos takes place in the tundra area of Alaska, Greenland and Siberia, *i.e.* in regions with a permanently frozen subsoil. Pingos are round to oval mounds with a core of ice and water. Although they are mostly much smaller, they can reach a diameter of more than 1000 m and a height of 100 m. The formation of these hills must be ascribed to the forces which come into play during the re-freezing of the water-logged topsoil thawed in summer. When, during the summers, the ice in the core melts off, the pingo collapses, and eventually a hollow is left in the soil. The depression is often surrounded by a ridge, the soliflucted material of the pingo skin (for literature see MAARLEVELD and VAN DEN TOORN 1955). Such a ridge has in fact been observed around some hollows in the ground moraine landscape of the northern Netherlands.

Terrain depressions formed during the Pleni-glacial would probably have been filled with sand within a comparatively short time. It would not have been until the transition from the Pleni-glacial to the Late-glacial that a more considerable deposition of organic material in pingo remnants began.

The depression filled with gyttja and peat from which was taken the profile to be discussed in this paper is situated 1.3 km northwest of the

[1]) Mr. P. Houtsma of Waskemeer kindly drew our attention to this deposit.

town of Waskemeer.[1]) 400 m south of the sampling spot lies the "Ganzemeer", one of the finest examples of a pingo remnant in this region (Fig. 1).

The Waskemeer diagram is composed according to the method introduced by IVERSEN (1942) for late-glacial diagrams. This diagram (Fig. 2) shows much resemblance to others published from the northern Netherlands (VAN DER HAMMEN 1949, 1951; DE PLANQUE 1949/50; WATERBOLK 1954; VAN ZEIST 1955). Consequently a general discussion of this diagram can be omitted and only some points will be touched upon.

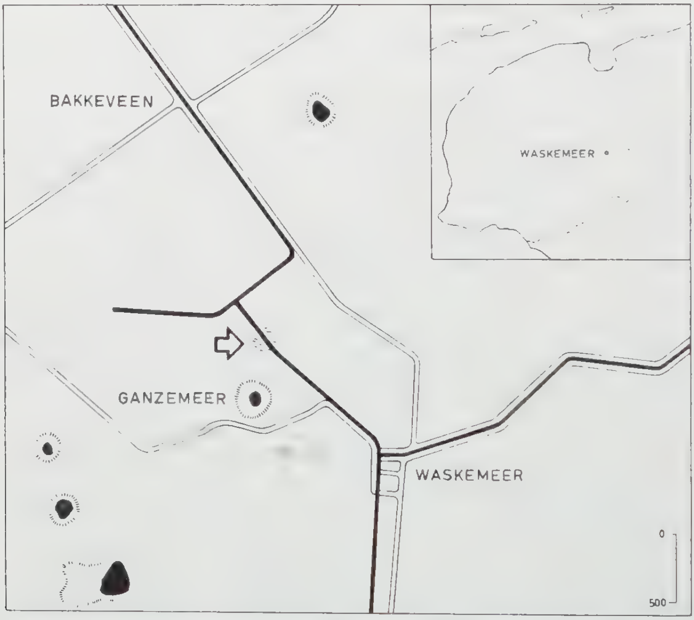


Fig. 1. Map of the Waskemeer region. The sampling spot is indicated by the arrow.

Zone II shows two *Pinus* maxima, suggesting a short deterioration of climate during the comparatively favourable Allerød time. As in several diagrams from Denmark *Betula* has a forked graph in Allerød time IVERSEN has already arrived at a tripartition of zone II, namely two relatively warm phases separated by a colder one (*cf.* KROG 1954). In the Waskemeer diagram this oscillation during the Allerød period would also be demonstrated for the northern Netherlands.

In the diagram of Fig. 2 the first *Pinus* maximum coincides with the presence of a small quantity of rebedded pollen of thermophilous trees. As with the exception of the lowermost samples pollen of certainly secondary origin is relatively scarce in this deposit it may be wondered whether the first *Pinus* maximum could be caused by rebedded *Pinus* pollen. The curves for *Pinus* and for the sum of the certainly secondary pollen (*Alnus*, *Quercus*, *Tilia*, *Ulmus*, *Picea*, *Juglans*)

in the lower part of the Waskemeer diagram (zones I, II and the beginning of III) are represented in Fig. 3. In contrast to those of Fig. 2 the pollen frequencies are shown here as percentages of the total tree pollen sum. The secondary pollen would for the greater part be derived from the boulder clay. As from this region no pollen analyses of boulder clay samples are available the correction method worked out by IVERSEN (1936) cannot be applied here. Nevertheless it is possible to determine whether the first *Pinus* maximum could be effected by contamination with rebedded pollen or not.

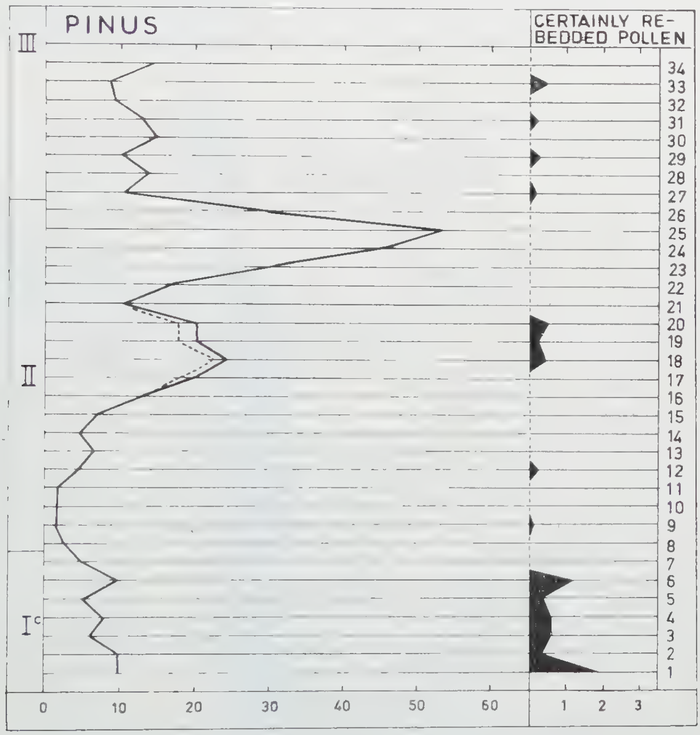


Fig. 2. For explanation see text.

As the amount of certainly secondary pollen is very low calculations carried out on the basis of this pollen will obviously be rather inaccurate. In the samples 1 to 7 (zone I) a total of 196 pollen grains of *Pinus* and 18 certainly rebedded ones were met with. In the samples 17 to 20 — the first *Pinus* maximum — 11 pollen grains of this last type were counted. Assuming that in the samples 1 to 7 all the *Pinus* pollen is of secondary origin the total number of rebedded *Pinus* pollen in the samples 17 to 20 can be calculated at $11/18 \times 196 = 120$, that is an average of 30 in each sample. The dotted line in Fig. 3 gives the *Pinus* percentages in the samples 17 to 20 corrected for the pollen of thermophilous trees and the rebedded *Pinus* pollen. Besides rebedded

Pinus pollen, *Betula* pollen of secondary origin would undoubtedly likewise be present. By subtracting the secondary *Betula* pollen from the tree-pollen sum already corrected for the rebedded pollen of *Pinus* and thermophilous trees, the values for *Pinus* will become somewhat higher than those represented by the dotted line. As nothing is known about the amount of rebedded *Betula* pollen this correction cannot be applied here.

Even in the case of too strong a correction for *Pinus* the curve for this tree clearly shows two maxima. In the Allerød part of other diagrams published from the northern Netherlands this course of the *Pinus* curve cannot be observed. This must probably be ascribed to the circumstance that in these diagrams zone II is composed of a relatively small number of spectra.

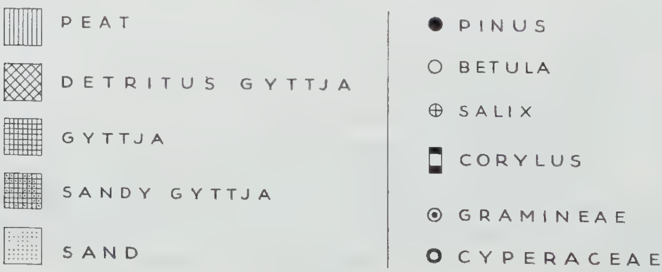
From *Epilobium angustifolium* five pollen grains were met with in zone III and one in the upper part of zone II. As a consequence of the numerous dead trees there would have been here many suitable habitats for this nitrophilous, light-demanding plant during the Late Dryas time.

As at present pollen of *Ephedra* is found in many late-glacial deposits from central and western Europe, the presence of *Ephedra cf. distachya* in the Late-glacial of the northern Netherlands comes wholly up to expectations. The circumstance that a pollen grain of *Ephedra* was also counted in zone II suggests that — at least locally — the vegetation would have been sufficiently open for this light-demanding plant.

The occurrence of *Typha latifolia* in zone III is more or less puzzling. In Scandinavia this plant does not extend beyond the 14° C July isotherm (HULTEN 1950; IVERSEN 1954), so that this would indicate a mean July temperature of at least 14° C in the northern Netherlands during the Late Dryas time. At present the mean July temperature in this region amounts to 16° C (BRAAK 1950). For that reason, on the basis of the conclusions arrived at by IVERSEN (1954) and others (*cf.* FIRBAS 1949), the mean July temperature during zone III must have been here considerably lower than 14° C, namely about 10–11° C.

During the zones I and II the vegetation of the lake itself — *Potamogeton, Myriophyllum alterniflorum, Isoetes lacustris, Lobelia, Equisetum* —

Fig. 3. Legend to the diagram



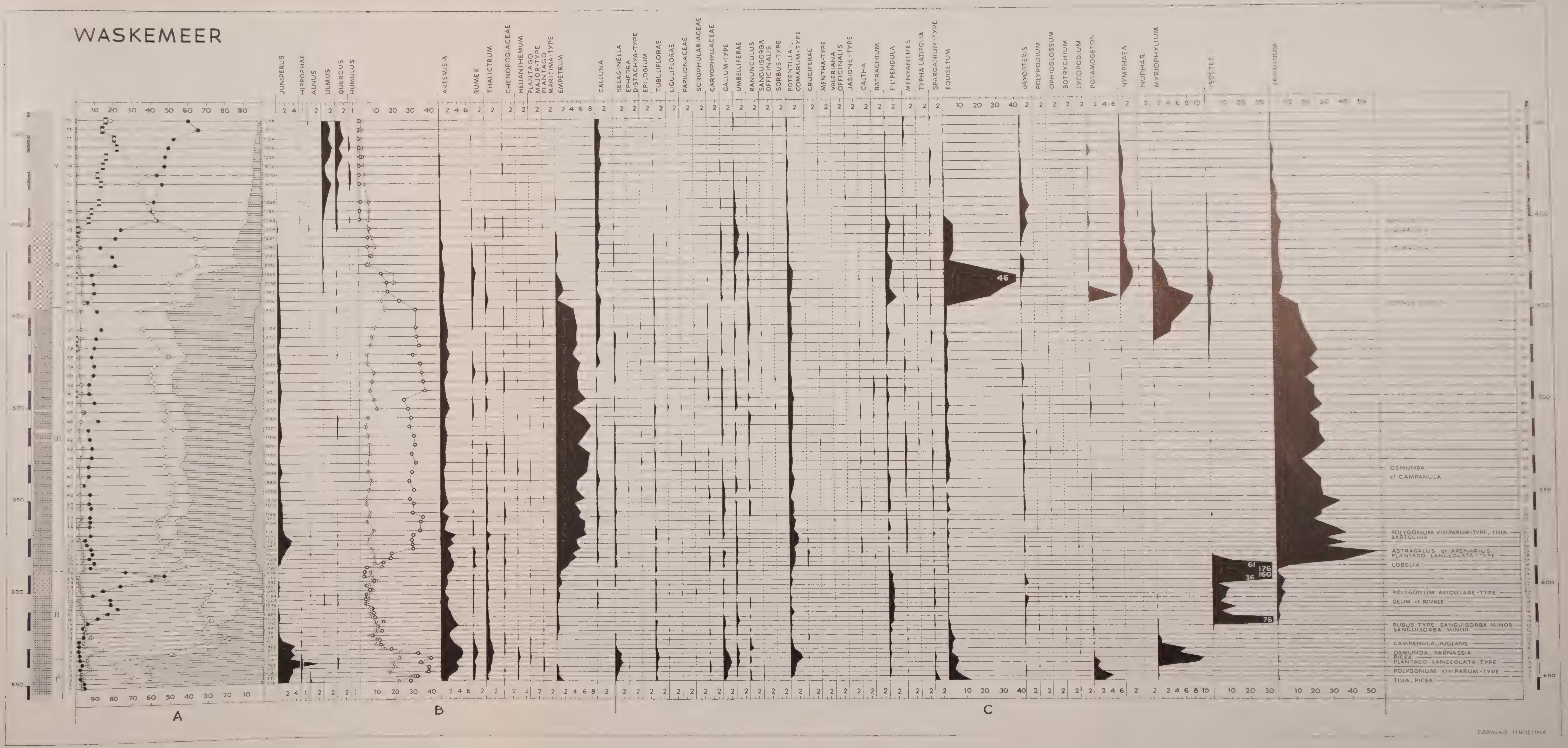

WASKEMEER
JUNIPERUS
HIPPOPHAË
ALNUS
ULMUS
QUERCUS
HUMULUS
ARTEMISIA
RUMEX
THALICTRUM
CHENOPODIACEAE
HELIANTHEMUM
PLANTAGO MAJOR-TYPE
PLANTAGO MARITIMA-TYPE
EMPETRUM
CALLUNA
SELAGINELLA
EPHEDRA DISTACHYA-TYPE
EPILOBIUM
TUBULIFLORAE
LIGULIFLORAE
PAPILIONACEAE
SCROPHULARIACEAE
CARYOPHYLLACEAE
GALIUM-TYPE
UMBELLIFERAE
RANUNCULUS
SANGUISORBA OFFICINALIS
SORBUS-TYPE
POTENTILLA-, COMARUM-TYPE
CRUCIFERAE
MENTHA-TYPE
VALERIANA OFFICINALIS
JASIONE-TYPE
CALTHA
BATRACHIUM
FILIPENDULA
MENYANTHES
TYPHA LATIFOLIA
SPARGANIUM-TYPE
EQUISETUM
DRYOPTERIS
POLYPODIUM
OPHIOGLOSSUM
BOTRYCHIUM
LYCOPODIUM
POTAMOGETON
NYMPHAEA
NUPHAR
MYRIOPHYLLUM
ISOËTES
SPHAGNUM
OSMUNDA
cf CAMPANULA
POLYGONUM VIVIPARUM-TYPE, TILIA
BARTSCHIA
ASTRAGALUS cf ARENARIUS
PLANTAGO LANCEOLATA-TYPE
LOBELIA
POLYGONUM AVICULARE-TYPE
GEUM cf RIVALE
RUBUS-TYPE, SANGUISORBA MINOR
SANGUISORBA MINOR
CAMPANULA, JUGLANS
OSMUNDA, PARNASSIA
PICEA
PLANTAGO LANCEOLATA-TYPE
POLYGONUM VIVIPARUM-TYPE
TILIA, PICEA
46
61
176
160
36
76
450
500
550
600
650
A
B
C
DRAWING H.ROELINK

Fig. 3

is typical for oligotrophic water and has to be assigned to the *Littorellion uniflorae* (*cf.* WESTHOFF *c.s.* 1946). The Late Dryas part of the diagram shows relatively high *Sphagnum* values, whereas from the plants mentioned above in general only a small number of pollen or spores was counted. In view of the character of the deposit — a sandy gyttja — there can have been no question of a small raised bog during zone III. It is probable that a vegetation of peat mosses growing in the water was concerned here. With regard to the marked decrease of *Potamogeton* and others, this could perhaps be ascribed to the deterioration of climate after the Allerød period only in the case of *Isoetes*. Thus in the diagrams from the Hijkermeer and the Mekelermeer (VAN DER HAMMEN 1949, 1951) high values for *Isoetes* can be observed both in zone II and zone IV. *Equisetum*, *Myriophyllum* and *Potamogeton* show fairly high values in zone I, so that the low percentages for these plants in zone III cannot be attributed to an unfavourable climate.

The relatively high percentages for *Sphagnum* contrasted with the low values for *Myriophyllum* and others could suggest that during zone III the water table in the lake was much higher than in the preceeding periods. In the central part of the depression the water would have become too deep for species such as *Equisetum* and *Myriophyllum*. These plants would have found suitable habitats on the border of the lake. In this connection it has to be remarked that the boring was carried out in the deepest part of the depression.

During the Late Dryas time a considerable sedimentation took place. This must have been effected to a large degree by the blowing in of sand. Between 5.00 and 5.20 m even some narrow sand layers were present. It seems that at the end of zone III the depth of the water had decreased even in the centre of the lake in such a way that *Myriophyllum alterniflorum* could thrive on the spot. The strong increase of *Equisetum* — probably *Equisetum fluviatile* — at the beginning of zone IV would have to be ascribed to the same circumstance.

The expansion of *Nymphaea alba* at the beginning of zone IV would have been the effect of the amelioration of climate. As for the vegetation of the lake during the Preboreal it can be remarked that a *Littorellion* in which *Nymphaea alba* as well as *Nuphar luteum* occurred was concerned here.

ACKNOWLEDGEMENT

The authors wish to thank Dr. J. J. Butler for the correction of the English text.

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Acta Botanica Neerlandica **9** (1960) 197–207

FLORISTISCHE NOTITIES 59–67

S. J. VAN OOSTSTROOM EN TH. J. REICHGELT
(*Rijksherbarium, Leiden*)

(*ingekomen 29 november 1959*)

Deze serie Floristische Notities [1]) bevat in hoofdzaak aanwinsten van de Nederlandse flora uit de jaren 1957 en 1958. De meeste hiervan werden reeds in het kort besproken in de lijsten van nieuwe planten in De Levende Natuur **61**, 1958, p. 134–135 en **62**, 1959, p. 162–163.

59. **Equisetum trachyodon** A.Br. (Fig. 1 en 2)

Asperen, op moerassig binnendijks terrein aan de noordzijde van de Linge, ten Z.O. van Asperen, leg. *Chr. G. van Leeuwen*, 7 juni 1957 (herb. L); idem, leg. *Unio*, 20 aug. 1957, herb. *v. O.* no. 20682 (herb. L).

E. trachyodon zou in augustus 1825 door Du Mortier (2, p. 371) gevonden zijn bij Beverwijk, maar is daar later nooit teruggevonden. In de bewerking van het geslacht *Equisetum* voor de Flora Neerlandica sprak de eerste van ons reeds het vermoeden uit (3, p. 31), dat Du Mortier een der beide op *E. trachyodon* gelijkende paardestaarten, die in het Duindistrict voorkomen, nl. *E. hyemale* L. var. *schleicheri* Milde of *E. variegatum* Schleich. ex Web. & Mohr voor *E. trachyodon* aangezien zou hebben. Dit was destijds door de oorlogsomstandigheden niet met zekerheid uit te maken, daar het herbarium van Du Mortier zich in Brussel bevindt.

Door de vondst bij Asperen werd onze aandacht weer op de soort gevestigd en kwamen wij ertoe het materiaal van Du Mortier te onderzoeken. Hierbij bleek dat Du Mortier's plant, volgens zijn etiket gevonden "vers Beverwijk entre Alkmar et Harlem le long du gd. chemin en traversant un petit bois" werkelijk *E. hyemale* var. *schleicheri* was, die ook nu nog vrij veel in het Duindistrict wordt aangetroffen.

Planten, die zeker tot *E. trachyodon* behoren, werden in vrij grote hoeveelheid op de boven aangegeven vindplaats bij Asperen gevonden, samen met *E. variegatum* en de var. *schleicheri* van *E. hyemale*.

E. trachyodon is in verschillende opzichten intermediair tussen deze beide soorten en wordt dan ook wel als een bastaard ervan beschouwd.

Het duidelijkste verschil tussen *E. variegatum* en *E. trachyodon* wordt gevonden in de vorm van de tanden der scheden (Fig. 1). Deze zijn bij *E. variegatum* eirond tot langwerpig lancetvormig, aan de top plotseling in een priemvormige spits versmald en hebben een brede witte, gladde rand. Bij *E. trachyodon* zijn de tanden lancet-priemvormig, geleidelijk naar de top versmald en hebben een smalle witte, ruwe rand. Bovendien sluiten de scheden bij *E. trachyodon* nauwer om de

[1]) Vroegere series werden gepubliceerd in A.B.N. **5**, 1956, p. 102–114; **5**, 1956, p. 322–334 en **7**, 1958, p. 33–52.

stengel dan bij *E. variegatum*. Verder zijn bij *E. trachyodon* de groeven tussen de ribben van de stengel ca. 2 tot 3 maal zo breed als de ribben, bij *E. variegatum* ongeveer 2 maal en is de stengel bij *E. trachyodon* gemiddeld forser.

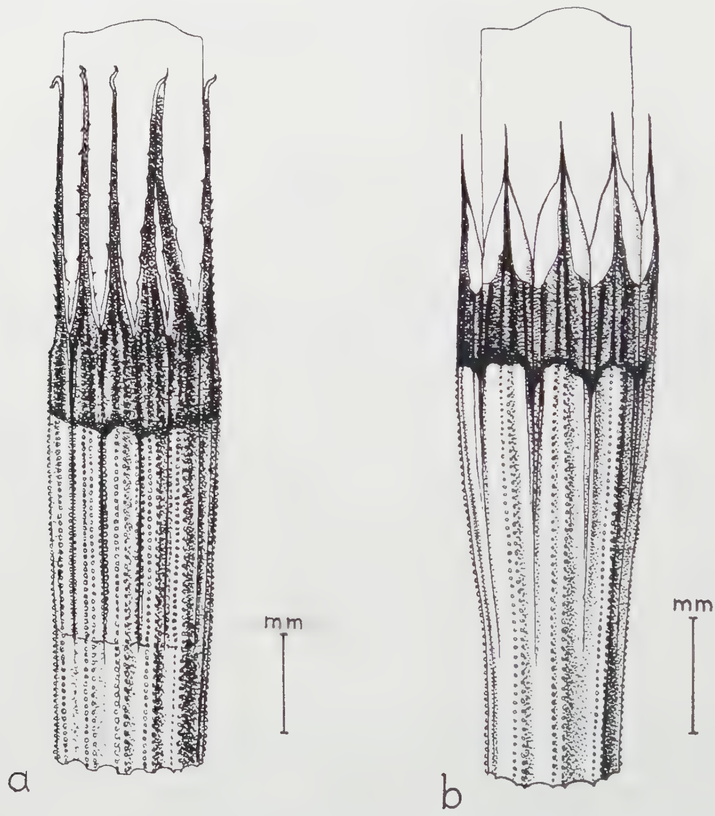

Fig. 1. Scheden van *a*: *Equisetum trachyodon* A. Br.; *b*: *E. variegatum* Schleich. ex Web. & Mohr (naar Luerssen).

Het duidelijkste verschil tussen *E. hyemale* en *E. trachyodon* is te constateren bij een dwarse doorsnede van de stengel (Fig. 2). De centrale holte neemt bij *E. hyemale* ca. $2/3$ deel of nog meer van de diameter van de stengel in, bij *E. trachyodon* slechts $1/4$ tot $1/3$ deel. Bovendien zijn de ribben bij *E. hyemale* vlak of iets gewelfd (zelden een weinig concaaf), bij *E. trachyodon* duidelijk concaaf, en zijn de groeven tussen de ribben bij de eerste 3 tot 3 $1/2$ maal zo breed als deze, bij *E. trachyodon* ca. 2 tot 3 maal. Verder vallen de tanden der scheden bij de typische var. van *E. hyemale* bijna steeds vroegtijdig af, terwijl ze bij *E. trachyodon* blijvend zijn of pas laat afvallen. Gemiddeld is de stengel van *E. trachyodon* minder fors dan die van *E. hyemale*.

De in het Fluviatiele en het Duindistrict voorkomende var. *schleicheri* van *E. hyemale* is iets moeilijker van *E. trachyodon* te onderscheiden dan

de typische var., omdat bij var. *schleicheri* de tanden van de scheden soms pas vrij laat afvallen en de plant ook vaak weinig forser is dan *E. trachyodon*. De bovengenoemde verschillen in de stengelbouw zijn echter steeds aanwezig.

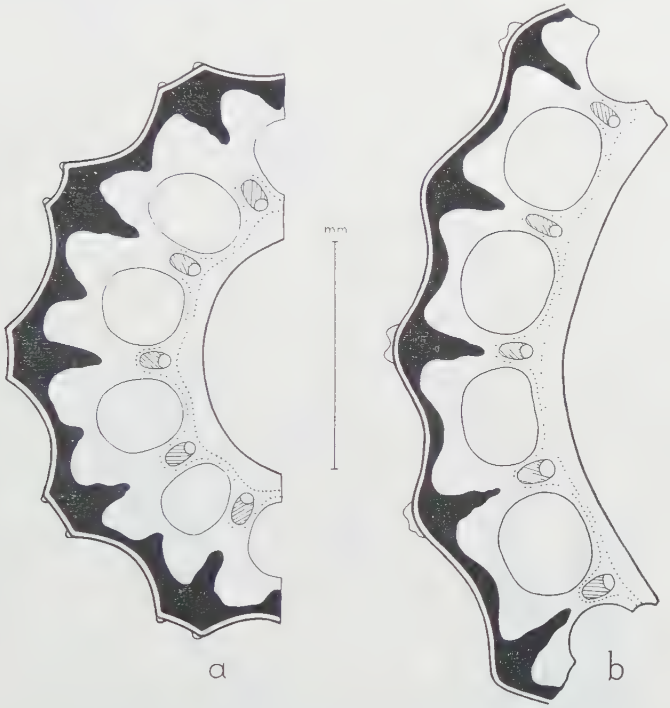

Fig. 2. Gedeelte van een dwarse doorsnede van de stengel van *a*: *Equisetum trachyodon* A. Br.; *b*: *E. hyemale* L. (naar Luerssen).

E. trachyodon is in Europa bekend van Zweden, Noorwegen, Schotland, Ierland, Z.W.-Duitsland (Bovenrijnse Laagvlakte), Zwitserland en Letland.

1. C. Luerssen, Die Farnpflanzen, Leipzig, 1889.
2. B. du Mortier, Bouquet de littoral Belge. Bull. Soc. Roy. Bot. Belg. **7**, 1868, p. 318–371.
3. S. J. van Ooststroom, Equisetaceae. Flora Neerl. **1** (afl. 1), 1948, p. 16–31.

60. **Caylusea abyssinica** (Fresen.) Fisch. & Mey. (Fig. 3, *b–d*)

Rotterdam, spoorwegterrein aan de Schiehaven, leg. *J. H. Kern, S. J. van Ooststroom* & *Th. J. Reichgelt*, 8 sept. 1958, herb. *v. O.* no. 21047 (herb. L).

Het tot de *Resedaceae* behorende geslacht *Caylusea* St.-Hil., dat slechts 3 soorten omvat en voorkomt op de Kaapverdische eilanden, in Noord- en Oost-Afrika, Arabië, Palestina en Perzië, onderscheidt zich van het geslacht *Reseda* L. in hoofdzaak als volgt:

Caylusea: vruchtbladen bijna tot aan de voet vrij, ieder met 2–3 basale, rechtopstaande zaadknoppen; vrucht bestaande uit 5–7 alleen

aan de voet samenhangende, open, kokervruchtachtige, stervormig uitgespreide delen (Fig. 3, *d*).

Reseda: vruchtbladen hoog vergroeid, met vele pariëtaal geplaatste,

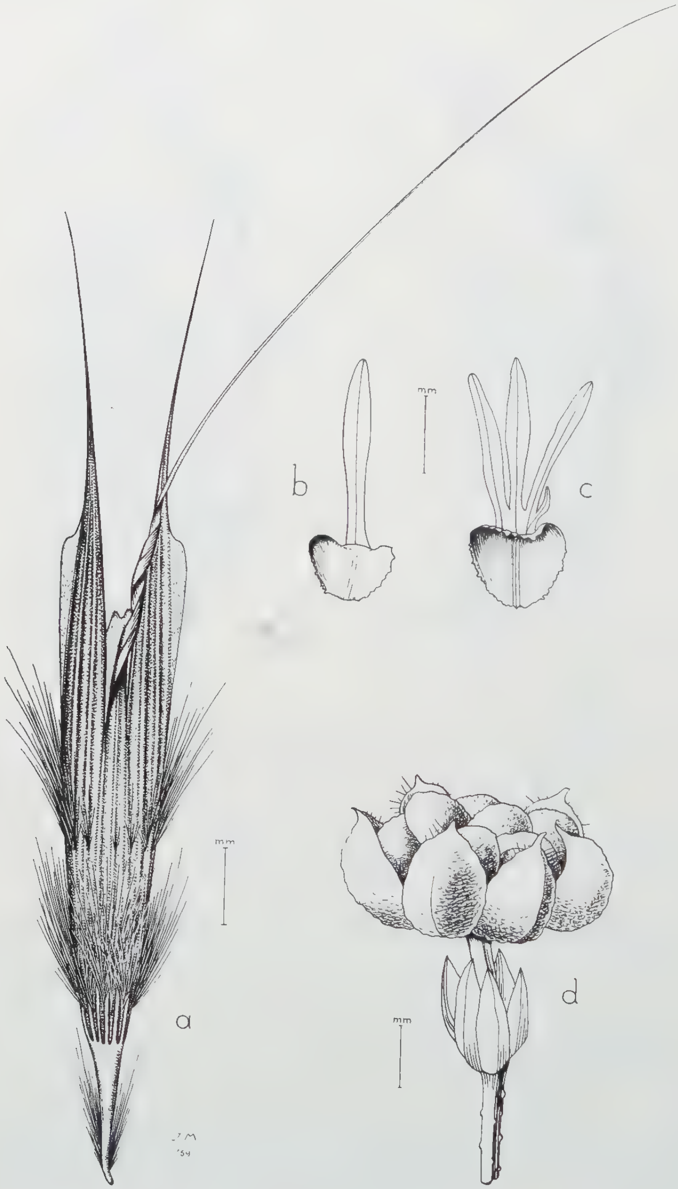

Fig. 3. *a*: *Danthonia racemosa* R. Br., bloem; *b–d*: *Caylusea abyssinica* (Fresen.) Fisch. & Mey., *b*: abaxiaal kroonblad, *c*: adaxiaal kroonblad, *d*: vrucht met kelk en androgynophoor (*b–d* naar Elffers & Taylor).

hangende zaadknoppen; vrucht een doosvrucht; deze alleen aan de top open, zeer zelden geheel gesloten, 2–5-lobbig.

C. abyssinica is als volgt gekenmerkt:

Plant kruidachtig, eenjarig (steeds?), in habitus gelijkend op *Reseda luteola* L. Stengel rechtopstaand of opstijgend, ca. 15–100 cm hoog, al of niet vertakt, kantig, vooral naar de top toe op de ribben iets wrattig of iets behaard. Bladen lancet- tot lijnvormig, met versmalde voet zittend, 3–12 cm lang, 2–18 mm breed, gaafrandig, soms iets gegolfd. Trossen eindelings, tijdens de bloei dicht, later verlengd, 5–40 cm lang; schutbladen lijn-priemvormig, ca. 3 mm lang, blijvend; bloemstelen tijdens de bloei iets korter dan de schutbladen, later weinig verlengd. Kelkbladen 5, lancetvormig, ca. 2–2½ mm lang, blijvend. Kroonbladen 5, wit, ca. 3–4 mm lang, de 2 adaxiale diep 4–5-spletig (Fig. 3, *c*), de 3 abaxiale ongedeeld of 2-spletig (Fig. 3, *b*), alle aan de voet met een omgekeerd driehoekig tot omgekeerd eirond, aan de randen kort gewimperd, schubvormig aanhangsel. Meeldraden 10–15, samen met de vruchtbladen op een androgynophoor; deze onder de inplantingsplaats van de meeldraden met een adaxiaal verbrede, schotelvormige discus, na de bloei verlengd. Helmdraden wit, papilleus; helmknoppen zalmkleurig of oranje, ten slotte geel. Kokervruchten met gewimperde rand. Zaden in totaal ca. 7–12, ongeveer niervormig, ca. 1,5 mm lang, wrattig-rimpelig, lichtbruin.

De soort komt voor in Oost-Afrika, van Eritrea en Ethiopië zuidelijk tot Tanganjika.

Een goede afbeelding ervan vindt men bij ELFFERS & TAYLOR (1, p. 2).

1. J. ELFFERS & P. TAYLOR, Resedaceae. Flora Trop. East Afr. 1958, p. 1–6.

61. **Ornithopus compressus** L. var. **leiocarpus** Eig

Bennekom, bosweg ten N. van en parallel met de Keijenbergse weg, op voor dennenaanplant afgerasterd terrein, leg. *A. N. Koopmans* & mej. *Joh. A. C. Veth*, 2 juni 1957 (herb. L).

Van *Ornithopus compressus* werd de typische variëteit, var. *compressus*, die behaarde peulen heeft, een tweetal malen hier te lande adventief aangetroffen en wel in 1917 op een rangeerterrein te Rotterdam (herb. L) en in 1918 op de ontginning Karelke bij Weert (herb. L). De opgave van een in 1874 aan de weg naar De Rith bij Breda gevonden plant (herb. NBV) heeft betrekking op een door OUDEMANS (in Ned. Kruidk. Arch. 2e ser., **2**, p. 153–154) ten onrechte tot deze soort gerekend exemplaar van *O. sativa* L.

De plant van Bennekom onderscheidt zich van de typische variëteit door het bezit van kale peulen en behoort tot var. *leiocarpus* Eig, in Inst. Agric. and Nat. Hist. Tel-Aviv, Bull. **6**, 1927, p. 26.

O. compressus komt voor door het gehele Middellandse Zee-gebied; de var. *leiocarpus* werd beschreven van Palestina.

62. **Linaria repens** (L.) Mill. × **L. vulgaris** Mill. (**L.** × **sepium** Allman).

Arnhem, aan de Koningsweg, leg. *C. G. Schippers*, sept. 1958 (herb. L).

Deze bastaard is in het gebied waar *Linaria repens* en *L. vulgaris* beide inheems zijn, d.i. in Zuid- en West-Europa, noordelijk tot Engeland en Frankrijk, herhaaldelijk aangetroffen. Zij werd aan de Koningsweg te Arnhem gevonden op een plaats waar *L. repens* en *L. vulgaris* beide voorkomen; de eerste, ter plaatse adventieve, soort zeker al sedert 1952, daar wij materiaal, dat in dit jaar verzameld werd, zagen in het herbarium van Dr. A. G. de Wilde te Sassenheim.

L. repens heeft een witte of licht lila bloemkroon, die donkerder geaderd is; deze adering is meestal op de beide bovenste kroonslippen het donkerst en op het masker het lichtst; het masker is aan de bovenzijde iets geel. De totale lengte van de bloemkroon bedraagt bij deze soort ca. 10–13 mm, waarvan de stompe spoor ca. 3 mm inneemt.

L. vulgaris heeft een bleekgele bloemkroon met een meestal oranje masker. De totale lengte van de bloemkroon bedraagt ca. 20–30 mm, waarvan de spitse spoor ca. 8–15 mm inneemt.

Het bij Arnhem gevonden exemplaar van de bastaard heeft een witte tot zeer licht lila bloemkroon, die lichter geaderd is dan bij *L. repens*; ook hier zijn de beide bovenste kroonslippen het donkerst geaderd. Het masker is zeer licht geel met een oranje vlek. De totale lengte van de bloemkroon bedraagt ca. 18–20 mm; de vrij spitse spoor is ca. 6–7 mm lang.

Wat de kleur en de afmetingen van de bloemkroon, de lengteverhouding van de totale bloemkroon en de spoor en ook wat de vorm van deze laatste betreft, houdt de plant dus wel ongeveer het midden tussen de beide ouders. Goed ontwikkelde vruchten waren aan het ons toegezonden, grotendeels reeds uitgebloeide materiaal niet te vinden.

63. **Ajuga pyramidalis** L.

Castricum, op een open, grazige plaats in de duinen ten W. van het dorp, in enige exemplaren, leg. *W. J. de Munk*, 23 juni 1958 (herb. L).

Deze soort wordt al sedert jaren in de Nederlandse flora's vermeld, uitsluitend naar aanleiding van een door L. Vuyck in april 1898 in de Leidse Hortus als opslag aangetroffen exemplaar (zie ook Prodr. Fl. Bat. ed. 2, I, **3**, 1904, p. 1346). Zij wordt bij ons een enkele maal als sierplant in tuinen gekweekt; de bij Castricum gevonden planten stonden ver van tuinen e.d. Het is niet onmogelijk, dat zij met fazantenvoer werden aangevoerd.

64. **Ligustrum vulgare** L. f. **chlorocarpum** (Loud.) Schelle en f. **xanthocarpum** (G. Don) Schelle

Een exemplaar van *Ligustrum vulgare* met groenblijvende bessen werd gevonden tussen talrijke andere met normale zwarte vruchten in de duinen van Westenschouwen in oktober 1957 door N. Lijssen. Het behoort tot f. *chlorocarpum* (Loud.) Schelle, in Beissner, Handb. Laubh. 1903, p. 419.

Een plant met gele bessen, f. *xanthocarpum* (G. Don) Schelle, l.c., werd aangetroffen bij het Quackjeswater te Rockanje op 28 december 1947 door C. Sipkes en door deze gedemonstreerd op de Kerstvergadering-1947 van de Commissie voor het Floristisch Onderzoek.

65. **Agoseris grandiflora** (Nutt.) Greene (Fig. 4)

'S-Heer Abtskerke, Zeel., berm langs een nieuwe weg, tussen nieuw ingezaaid gras, samen met *Madia sativa* Molina, leg. *A. de Visser*, 27 juli en 3 sept. 1958 (herb. L).

Het Composieten-geslacht *Agoseris* Raf. behoort tot de tribus *Cichorieae*, subtribus *Crepidinae*, waartoe van de hier te lande voorkomende geslachten ook *Hieracium*, *Crepis*, *Sonchus*, *Lactuca* en *Taraxacum* gerekend worden.

Van *Hieracium* verschilt het o.a. door de aan de top niet afgeknotte, doch versmalde of meestal gesnavelde vruchten, van *Crepis*, *Sonchus* en *Lactuca* door de steeds onbebladerde eenhoofdige bloeistengels, van de beide laatste geslachten bovendien door de niet samengedrukte,

Fig. 4. *Agoseris grandiflora* (Nutt.) Greene; *a*: plant; *b*: vrucht.

rolronde of kantige vruchten, van *Taraxacum* ten slotte in hoofdzaak door de bij de top niet gestekelde vruchten.

Agoseris vertoont in habitus een zekere gelijkenis met sommige soorten van het tot de tribus *Cichorieae*, subtribus *Leontodontinae* behorende geslacht *Scorzonera*. Dit laatste wijkt echter af door het bezit van een, althans bij de middelste bloemen van het hoofdje, geveerd pappus.

A. grandiflora, die afkomstig is uit westelijk Noord-Amerika, waar de soort gevonden wordt van Brits Columbia tot Californië, is als volgt te karakteriseren:

Overblijvend, melksap bevattend kruid, in de jeugd meestal een weinig behaard, later vrijwel kaal behalve aan de voet van het omwindsel, waar een blijvende min of meer witwollige beharing voorkomt. Stengel ca. 20–70 cm hoog, onvertakt, eenhoofdig. Bladen alle in een wortelrozet, omgekeerd lancet- tot lijn-lancetvormig, gaafrandig tot veerdelig, ca. 10–30 cm lang. Hoofdjes met uitsluitend lintbloemen; omwindsel tijdens de bloei ca. 1½–2, na de bloei tot 4 cm hoog; buitenste omwindselbladen vooral aan rand en voet wollig behaard, eirond tot langwerpig eirond, korter dan de minder behaarde tot kale, veel smallere binnenste. Bloemen weinig langer dan het omwindsel, geel. Achenen (Fig. 4, *b*) smal spoelvormig, ca. 6 mm lang, geribd, met ongeveer 10 ribben, geleidelijk versmald in de 2–4 maal zo lange snavel; pappus uit enkelvoudige haren bestaand, wit.

66. **Danthonia racemosa** R.Br. (Fig. 3, *a*)

Helmond, terrein bij dekenfabriek De Wit, leg. *A. W. Kloos Jr*, 22 sept. 1939 (herb. L); Tilburg, terrein bij Tilburgse Wolwasserij, leg. *A. W. Kloos Jr* & *Th. J. Reichgelt*, 4 sept. 1951 (herb. L); id., terrein bij wolfabriek Bern. Pessers, leg. *J. H. Kern, S. J. van Ooststroom* & *Th. J. Reichgelt*, 1 sept. 1958, herb. *v.O.* no. 21029 (herb. L).

Het boven geciteerde exemplaar van Helmond werd door Kloos (3, p. 125) ten onrechte als *Danthonia compressa* Aust. vermeld. Ook Jansen (2, p. 192) vermeldt het onder deze naam. De in 1951 bij Tilburg gevonden plant werd door Kloos en Van Ooststroom (4, p. 176) in hun lijst van voor Nederland nieuwe soorten op gezag van Jansen met enige twijfel als *D. intermedia* Vasey opgegeven.

Bij een nader onderzoek bleek ons, dat beide planten en ook de in 1958 bij Tilburg verzamelde niet behoren tot de genoemde soorten, die beide inheems zijn in Noord-Amerika, maar dat wij te maken hadden met een Australische soort. Eerst dachten wij, dat dit *D. semiannularis* (Labill.) R. Br. was, onder welke naam wij het adventieve materiaal vermeldden in De Levende Natuur (5, p. 163). Na bestudering van de bewerking van Vickery (6) bleek ook deze opvatting onjuist en kwamen wij tot het resultaat, dat wij te doen hadden met *D. racemosa* R. Br.

D. racemosa verschilt van de beide Amerikaanse soorten vooral door de beharing van het onderste kroonkafje. Dit is bij *D. compressa* over de gehele oppervlakte, behalve aan de top, verspreid behaard, terwijl het bij *D. intermedia* alleen in het onderste deel aan de randen behaard is. Bij *D. racemosa* daarentegen vertoont het onderste kroonkafje (Fig.

3, *a*) een dwarse band van haren boven de voet en daarboven, tussen deze band en de insnijding van het kafje, een haarbosje ter weerszijden aan de rand, bovendien op dezelfde hoogte soms nog enkele kleinere haarbosjes op de rug; overigens is het kaal. Voorts is bij deze soort het callus tot ruim 1 mm lang, terwijl het bij *D. compressa* en *D. intermedia* ten hoogste ca. ½ mm lang is.

D. semiannularis heeft een behaard onderst kroonkafje, dat de band van lange haren bij de voet mist, terwijl onder de insnijding een dwarse band van haren voorkomt, die langer zijn dan de haarbosjes bij *D. racemosa*.

Vergelijkt men de afbeelding bij JANSEN (2, p. 192, fig. 146) met een aartje van de Helmondse plant, dan is het duidelijk, dat deze niet naar genoemde plant getekend is. Veeleer is de tekening een kopie van de afbeelding, die HITCHCOCK (1, p. 309, fig. 416) geeft van een aartje van *D. spicata* (L.) P.B. ex R. & S., een veel op *D. compressa* gelijkende soort, waarbij de zijdelingse tanden van het onderste kroonkafje wat langer getekend zijn dan bij de figuur van *D. spicata* van Hitchcock het geval is, waarschijnlijk om ze op die van *D. compressa* te doen gelijken.

Een uitvoerige beschrijving van *D. racemosa* is te vinden bij VICKERY (6, p. 316).

1. A. S. HITCHCOCK, Manual of the grasses of the United States, ed. 2, Washington, 1951.
2. P. JANSEN, Gramineae. Flora Neerl. **1**, afl. 2, 1951.
3. A. W. KLOOS Jr, Aanwinsten van de Nederlandse flora in 1939. Ned. Kruidk. Arch. **50**, 1940, p. 123–145.
4. A. W. KLOOS JR & S. J. VAN OOSTSTROOM, Nieuwe plantensoorten en -vormen in Nederland gevonden in 1951. De Levende Natuur **55**, 1952, p. 176.
5. S. J. VAN OOSTSTROOM & TH. J. REICHGELT, Nieuwe plantesoorten in Nederland gevonden in 1958 en vroegere jaren. De Levende Natuur **62**, 1959, p. 162–163.
6. J. W. VICKERY, A revision of the Australian species of Danthonia DC. Contr. New South Wales Nat. Herb. **2**, 1956, p. 249–325, 6 pl.

67. **Verwilderde cultuurplanten**

a. **Pachysandra terminalis** Sieb. & Zucc.

Gronsveld, Limb., op een lommerrijke plaats buiten een tuin, leg. *B. Brinkman*, 26 april 1958 (herb. L).

Deze in tuinen en parken gekweekte *Buxacea*, afkomstig uit Japan, werd op bovengenoemde plaats verwilderd aangetroffen.

b. **Thalictrum dipterocarpum** Franch.

Oostvoorne, leg. *F. J. Pronk*, 4 juli 1958 (herb. L).

Th. dipterocarpum wordt wel als borderplant in tuinen gekweekt. Zij is afkomstig uit West-China en werd bij Oostvoorne ver van tuinen e.d. aangetroffen.

c. **Rubus crataegifolius** Bunge

Wenum, gem. Apeldoorn, Kopermolenweg, beschaduwde wegkant, leg. *A. N. Koopmans* & mej. *Joh. A. C. Veth*, 1 aug. 1958 (herb. Koopmans).

R. crataegifolius, afkomstig uit Noord-China en Japan, behoort tot

het subgenus *Idaeobatus* Focke. De soort wordt bij ons zelden als sierplant gekweekt.

d. **Staphylea pinnata** L.

St. Geertruid, Limb., in het Eisder Bos, leg. *I.V.O.N.-exc.*, 26 mei 1958, herb. *v.O.* no. 20793 (herb. L).

Deze uit Midden- en Zuid-Europa afkomstige sierheester kwam op een plek in het Eisder Bos in een aantal exemplaren voor en maakte de indruk daar geheel verwilderd te zijn.

e. **Anchusa capensis** Thunb.

's-Gravenhage, Kwekerijweg, tussen het gras, leg. mej. *J. Th. Koster*, 6 aug. 1931 (herb. L); stortterrein tussen Wartena en Eernewoude, Fr., leg. *D. Franke* & *D. T. E. van der Ploeg*, 1956 (herb. v. d. Ploeg).

Een uit Zuid-Afrika afkomstige, als borderplant gekweekte soort, die in enkele kleurvariëteiten voorkomt. De hierboven geciteerde exemplaren hadden hemelsblauwe bloemen.

f. **Mertensia ciliata** (James ex Torr.) G. Don

Velsen, leg. *F. W. Burger*, 23 juni 1914 (herb. NBV); IJpolder, leg. *Van Heusden*, 29 juni 1915 (herb. NBV).

De hierboven geciteerde exemplaren werden in de Nederlandse flora's ten onrechte als de uit Siberië afkomstige *Mertensia sibirica* (L.) G. Don vermeld. Zij behoren echter tot de in de westelijke Verenigde Staten inheemse, bij ons meer dan de eerstgenoemde soort als borderplant gekweekte *M. ciliata*, die zich van *M. sibirica* o.a. onderscheidt door de gewimperde bladrand en kelkslippen. Ook Boom (1, p. 210) constateert, dat planten, die onder de naam *M. sibirica* in cultuur zijn, vaak tot *M. ciliata* behoren.

1. B. K. Boom, Flora der Cultuurgewassen van Nederland **2**, Wageningen, 1950.

g. **Symphytum asperum** Lepech.

Bij Utrecht, leg. *A. C. Oudemans*, 23 mei 1879 (herb. NBV).

Verscheidene malen werd in ons land adventief of verwilderd de bastaard van *Symphytum asperum* Lepech. en *S. officinale* L. (*S.* × *uplandicum* Nyman) aangetroffen. Van *S. asperum* zelf zagen wij tot voor kort geen Nederlandse exemplaren. Wel vermeldt De Wever (1, p. 115 en 116), dat deze soort op enige plaatsen in Zuid-Limburg uit de cultuur als sierplant verwilderd zou zijn waargenomen en dat zij ook wel eens voorkwam als verontreiniging in de aldaar sedert 1920 opgegeven cultuur van *S.* × *uplandicum*, maar in zijn herbarium in het Natuurhistorisch Museum te Maastricht troffen wij geen materiaal ervan aan.

Bij het doorzien van de exemplaren van *S. officinale* in de collectie van het Rijksherbarium vonden wij onlangs het hierboven geciteerde exemplaar, dat door Oudemans niet als zodanig herkend was.

1. A. de Wever, Symphytum asperrimum Don en S. uplandicum Nym. Natuurhist. Maandbl. Limb. **21**, 1932, p. 115–116.

h. **Galanthus elwesii** Hook. f.

Wassenaar, Zuidwijk, leg. *H. J. Lam, D. Bakker* & *L. B. Holthuis* no. 6723, 5 april 1941 (herb. L).

G. elwesii, die reeds in 1941 als verwilderde sierplant in het bos van de buitenplaats Zuidwijk bij Wassenaar werd gevonden, bleek daar in 1958 nog steeds voor te komen, blijkens een vondst door de eerste van ons op 22 maart van dat jaar.

De soort wordt oorspronkelijk gevonden in Klein-Azië en op het Balkan Schiereiland.

i. **Iris sibirica** L.

Amerongen, Overberg, in een spoorsloot, leg. *M. T. Jansen*, 7 juni 1958 (herb. L).

Een in een groot deel van gematigd Europa en Azië, oostelijk tot in Japan voorkomende soort, die bij ons vaak als sierplant wordt gekweekt.

Behalve op bovengenoemde vindplaats trof Jansen haar reeds in 1947 aan bij de molenvijver van de Plasmolen, Limb., waar zij echter nu verdwenen is.

SUMMARY

With a few exceptions this series of "Floristische Notities" contains additions to the flora of the Netherlands from the years 1957 and 1958.

It consists of the following notes:

59. *Equisetum trachyodon* A. Br., found for the first time in the Netherlands near Asperen (Gelderland) on marshy soil along the river Linge. Du Mortier mentioned this species already for our country in 1825, but his specimens, in the herbarium at Brussels, appeared to belong to *E. hyemale* L. var. *schleicheri* Milde. Differences between *E. trachyodon* and *E. variegatum* Schleich. ex. Web. & Mohr mainly as to the shape of the teeth of the sheaths and between *E. trachyodon* and *E. hyemale* L. as to the anatomical structure of the stem are mentioned and illustrated.

60, 61, 63, 65, and 66. *Caylusea abyssinica* (Fresen.) Fisch & Mey., *Ornithopus compressus* L. var. *leiocarpus* Eig, *Ajuga pyramidalis* L., *Agoseris grandiflora* (Nutt.) Greene, and *Danthonia racemosa* R. Br. First records of these aliens for the Netherlands, the last one near wool factories at Helmond and Tilburg.

62. A hybrid between the native *Linaria vulgaris* Mill. and the adventive *L. repens* (L.) Mill. was found near Arnhem, with the putative parents.

64. Two forms of *Ligustrum vulgare* L., one with green fruits (f. *chlorocarpum*) and the other with yellow ones (f. *xanthocarpum*). Both found in the dunes, the former near Westenschouwen (Zeeland), the latter near Rockanje (Zuid-Holland).

67. A number of plants escaped from cultivation.

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ON THE OCCURRENCE OF AMYLOIDS IN PLANT SEEDS [1])

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INTRODUCTION

Schleiden (1838) and Vogel and Schleiden (1839) were the first to observe a blue coloration of the cell-walls in the cotyledons of some plant species when the latter were treated with an iodine-potassium iodide solution. The cell-wall substance which coloured blue with this reagent, was called "amyloid" by these authors.

Afterwards several investigators found this amyloid in the cell-walls of cotyledons and of endosperm, viz. Trécul (1858), Frank (1866), Heinricher (1888), Reiss (1889), Nadelmann (1890), Winterstein (1893), Van Wisselingh (1898). These authors observed the substance in seeds of the species listed in Table I.

TABLE I

Occurrence of amyloid in seeds according to earlier authors.

Acanthaceae	*Primulaceae*
Schwabea	Anagallis arvensis
Balsaminaceae	Androsace septentrionalis
Impatiens balsamina	Cyclamen europaeum
Leguminosae-Papilionatae	,, neapolitanum
Goodia lotifolia	Glaux maritima
Mucuna urens	Primula officinalis
Leguminosae-Caesalpinioideae	Samolus valerandi
Hymenaea courbaril	*Ranunculaceae*
Schotia latifolia	Paeonia officinalis
,, speciosa	*Sapotaceae*
Tamarindus indica	Dipholis
Linaceae	Mimusops balata
Linum usitatissimum	Sideroxylon
Myrsinaceae	*Thunbergiaceae*
Ardisia	Thunbergia alata
	Tropaeolaceae
	Tropaeolum majus

Some of these authors found amyloid also in seeds of some other plant species, chiefly in those of *Monocotyledones*, but their results could not be confirmed by other investigators. It seems possible that not all these authors applied the same reagent, but this can not be proved directly, because several investigators did not record the composition of their reagent.

Mitchell (1930) investigated the influence exercised on the colora-

[1]) This work is part of a doctor's thesis (Delft, 1959).

tion of cell-walls by the addition of sulphuric acid to the iodine-potassium iodide solution, and stated that the cell-walls in the seeds of *Tamarindus indica* were already stained blue by the reagent without addition of sulphuric acid. The cell-walls of seeds of *Impatiens balsamina*, of *Primula officinalis* and of *Tropaeolum majus* stained violet when after the iodine-potassium iodide solution a solution of 26 volume percent sulphuric acid was added. After the addition of 50 volume percent sulphuric acid the cell-walls in the endosperm of *Coffea arabica*, of *Diospyros virginiana*, of *Phoenix dactylifera* and of *Strychnos nux-vomica*, and in the cotyledons of *Lupinus hirsutus* also stained blue. Evidently the composition of the reagent is of great influence upon the results.

In the earlier publications where a positive amyloid reaction was postulated for the seeds of a number of *Monocotyledones*, the reagent (apart from differences in the iodine and KJ concentrations) must have contained a fair quantity of sulphuric acid or some other swelling agent. In my own experiments MITCHELL's reagent when applied without any addition, gave no coloration of cell-walls in the seeds of any *Monocotyledon*. In view of the diverging results obtained with reagents of different composition, it is important to specify the reagent used for the amyloid reaction.

In my experiments the cell-walls in the seeds of *Impatiens balsamina*, of *Primula officinalis* and of *Tropaeolum majus* already stained blue with MITCHELL's reagent when the latter was applied without the addition of sulphuric acid. This difference is caused by the fact that MITCHELL soaked the seeds before applying the reagent, while I applied the reagent to sections of untreated seeds. In MITCHELL's experiments the reagent penetrated into the sections in a diluted form.

For this reason I adopted as criterion for a positive reaction on the amyloid in the cell-walls the development of a blue or violet colour when to untreated, dry sections a solution was added which contained 0.3 g iodine and 1.6 g potassium iodide per 100 ml water. This is MITCHELL's reagent without sulphuric acid.

When defined in this way, the amyloids occurring in plant seeds appear to constitute a group of polysaccharides with a closely related structure.

This investigation was undertaken in order to obtain an impression of the occurrence of amyloids in plant seeds, and to see whether some correlation could be detected between the occurrence of these substances and the taxonomic position of the plant species.

This paper gives a full record of a number of experiments on which a short communication appeared some time ago (KOOIMAN, 1957).

METHOD

The amyloid reaction was carried out by adding a drop of the reagent to some sections of a cotyledon or of the endosperm of the plant under investigation. Very small seeds were crushed, and then mixed with a drop of the reagent.

Colouring of the cell-walls was observed microscopically.

RESULTS AND DISCUSSION

A. Leguminosae-Caesalpinioideae

In Table II all the genera of the tribes *Cynometreae*, *Amherstieae* and *Sclerolobieae* of the *Leguminosae-Caesalpinioideae* are listed alphabetically. Behind the name of each genus the number of investigated species is given; a positive sign means a positive amyloid reaction, a negative sign means a negative amyloid reaction.

TABLE II

Results of amyloid reaction in seeds of *Leguminosae – Caesalpinioideae – Tribes Cynometreae – Amherstieae – Sclerolobieae* [1]) (alphabetical)

Afzelia	4+	Eperua	1+	Paramacrolobium	1+
Amherstia	1+	Eurypetalum		Pellegriniodendron	
Amphimas	1—	Gilbertiodendron	1+	Peltogyne	3+
Anthonotha	3+	Gilletiodendron	1+	Phyllocarpus	1+
Apaloxylon		Goniorrhachis		Plagiosiphon	1+
Aphanocalyx	2+	Gossweilerodendron	1—	Poeppigia	1—
Augouardia		Guibourtia	1+	Polystemonanthus	
Baikiaea	2+	Hardwickia	1—	Prioria	
Batesia	1—	Heterostemon		Pterogyne	1—
Bathiaea		Humboldtia	1+	Pseudocopaiva	
Berlinia	3+	Hylodendron	1+	Pseudomacrolobium	
Brachystegia	2+	Hymenaea	4+	Pseudosindora	
Brownea	1+	Hymenostegia	2+	Recordoxylon	
Browneopsis		Intsia	2+	Saraca	1+
Campsiandra	1—	Isoberlinia	3+	Schotia	4+
Cenostigma		Julbernardia	3+	Sclerolobium	3—
Colophospermum	1—	Kingiodendron	1—	Scorodophloeus	1+
Copaifera	8+	Lebruniodendron		Sindora	4+
Crudia	2+	Librevillea		Sindoropsis	
Cryptosepalum	2+	Loesenera		Stahlia	1—
Cymbosepalum		Lysidice		Stemonocoleus	
Cynometra	4+	Macrolobium	2+	Tachigalia	2—
Daniellia	2+	Maniltoa	1+	Talbotiella	
Detarium	1+	Melanoxylon	1—	Tamarindus	1+
Dicymbe	2+	Michelsonia		Tessmannia	1+
Dicymbopsis		Microberlinia		Tetraberlinia	
Diptychandra		Monopetalanthus	1+	Thylacanthus	
Didelotia		Neochevalierodendron		Trachylobium	1+
Elizabetha	1+	Oddoniodendron		Vouacapoua	
Endertia	1+	Oxystigma	2—	Zenkerella	
Englerodendron		Palovea	1+		

Table III records the species with a positive amyloid reaction belonging to the genera listed in Table II.

In Table IV the genera of the other tribes of the *Leguminosae-Caesalpinioideae* are listed. Here the result was always negative.

Whereas previously but four species were known to have amyloid in the cell-walls of the cotyledons (see Table I), this substance now appeared to occur much less exceptionally in this sub-family than could have been anticipated. About 90 species belonging to 43 genera were found to have amyloid.

[1]) The figures indicate the number of species that were investigated; + positive, — negative reaction.

TABLE III

Leguminosae-Caesalpinioideae

Amyloid-containing species

Afzelia africana (Sm.) Pers.
bella Harms
bipindensis Harms
javanica (Miq.) J. Léonard
quanzensis Welw.
Amherstia nobilis Wall.
Anthonotha gilletii (De Wild.) J. Léonard
macrophylla P. Beauv.
pynaertii (De Wild.) Exell et Hillcoat
Aphanocalyx cynometroides Oliv.
spec. (Flora Congo III, 440)
Baikiaea insignis Benth. subspec. insignis J. Léonard
subspec. minor (Oliv.) J. Léonard
eminii Taub.
Berlinia giorgii De Wild.
var. gilletii (De Wild.) Hauman
grandiflora (Vahl) Hutch. et Dalz.
viridicans Bak. f.
Brachystegia stipulata De Wild.
var. lufirensis (De Wild.) Hoyle
spec.
Brownea spec.
Copaifera baumiana Harms
coriacea Mart.
duckei Dwyer
epunctata Amshoff
guianensis Desf.
mildbraedii Harms
officinalis L.
reticulata Ducke
Crudia glaberrima (Steud.) Macbr.
harmsiana De Wild.
Cryptosepalum maraviense Oliv.
spec.
Cynometra alexandri C. H. Wright
ananta Hutch. et Dalz.
leonensis Hutch. et Dalz.
sessiliflora Harms
Daniellia alsteeniana Duvign.
oliveri (Rolfe) Hutch. et Dalz.
Detarium senegalense J. F. Gmel.
Dicymbe altsoni Sandw.
corymbosa Spruce
Elizabetha speciosa Ducke
Endertia spectabilis Van Steenis et De Wit
Eperua bijuga Mart.
Gilbertiodendron demeusei (De Wild.) J.Léonard
Gilletiodendron mildbraedii (Harms) Verm.
Guibourtia coleosperma J. Léonard
Humboldtia laurifolia Vahl
Hylodendron gabunense Taub.
Hymenaea altissima Ducke
courbaril L.
oblongifolia Huber
parvifolia Huber
Hymenostegia afzelii Harms
laxiflora (Benth) Harms
Intsia amboinensis A.D.C.
bijuga O. Kuntze
Isoberlina angolensis (Welw.) Hoyle et Brenan
scheffleri (Harms) Greenway
tomentosa (Harms) Craib et Stapf
Julbernardia baumii (Harms) Troupin
paniculata (Benth.) Troupin
seretii (De Wild.) Troupin
Macrolobium bifolium (Aubl.) Pers.
multijugum Benth.
Maniltoa gemmipara Scheff.
Monopetalanthus pteridophyllus Harms
Palovea guianensis Aubl.
Paramacrolobium coeruleum (Taub.) J. Léonard
Peltogyne densiflora Spruce
pubescens Benth.
venosa (Vahl) Benth.
Phyllocarpus riedelii Tul.
Plagiosiphon emarginatus (Hutch. et Dalz.) J. Léonard
Saraca indica L.
Schotia bequaertii (De Wild.) De Wild.
brachypetala Sond.
latifolia Jacq.
speciosa Jacq.
Scorodophloeus zenkeri Harms
Sindora cochinchinensis Baill.
irpicina De Wit
klaineana Pierre et Pellegr.
sumatrana Miq.
Tamarindus indica L.
Tessmannia africana Harms
Trachylobium verrucosum Oliv.

Amyloid is strictly confined to the three tribes of Table II which are taxonomically nearly related.

The classification of the genera in the tribes *Cynometreae, Amherstieae* and *Sclerolobieae* has always presented difficulties. As recent examples of diverging opinions the views of DWYER (1954b) and of LÉONARD

TABLE IV

Results of amyloid reaction in the tribes of the *Leguminosae-Caesalpinioideae* (except *Cynometreae*, *Amherstieae* and *Sclerolobieae*) [1])

4. *Bauhinieae*		
Bandeiraea	2—	
Bauhinia	3—	
Cercis	2—	
Gigasiphon		
5. *Cassieae*		
Apuleia	2—	G
Baudouinia		
Cassia	3—	G
Ceratonia	1—	G
Dansera		
Dialium	4—	
Dicorynia	2—	
Distemonanthus	1—	
Koompassia	2—	
Labichea	1—	
Martia	1—	G
Oligostemon	1—	S
Petalostylis	1—	
Storckiella	1—	
Stuhlmannia		
Uittienia		
6. *Dimorphandreae*		
Brandzeia		
Burkea	1—	
Chidlowia	1—	S
Dimorphandra	3—	
Erythrophloeum	1—	
Kaoue		
Mora		
Pachyelasma	1—	
Sympetalandra		
7. *Eucaesalpinieae*		
Acrocarpus	2—	
Aprevalia		
Bussea	1—	S
Caesalpinia	4—	G
Cercidium	2—	G
Colvillea	1—	G
Delonix	1—	G
Gleditschia	1—	G
Gymnocladus	1—	G
Haematoxylon	1—	G
Hoffmanseggia	2—	
Jaqueshuberia		
Mezoneurum	1—	
Moldenhawera	1—	
Parkinsonia	1—	G
Peltophorum	1—	
Pogocybe		
Pterolobium	1—	
Schizolobium	1—	G
Stachyothyrsus		
Wagatea	1—	
Zuccagnia		
8. *Kramerieae*		
Krameria	2—	
9. *Swartzieae*		
Aldina		
Baphiopsis	1—	
Cordyla	1—	S
Exostyles	1—	S
Holocalyx	1—	S
Lecointea		
Mildbraediodendron	1—	S
Swartzia	10—	S
Zollernia	2—	S

(1959) may be mentioned. DWYER suggests to unite the three tribes, because in his opinion the characters by means of which BENTHAM (1865) had tried to delimit the latter, are not of essential value. However, LÉONARD, in dealing with the *Amherstieae* and *Cynometreae* of Africa, judges it advantageous to keep these tribes apart, but he introduces other characters to define them. BAKER (1926–1930) united these two tribes, but he made a subdivision of the enlarged tribe; LÉONARD's *Amherstieae* fall in the first part while the second and third parts correspond to LÉONARD's *Cynometreae*.

Of BENTHAM's *Sclerolobieae* only *Dicymbe* and *Phyllocarpus* appeared to contain amyloid. DUCKE, the great authority on the Brazilian *Caesalpinioideae*, (1949) classifies *Dicymbopsis* and *Tachigalia* in the *Amherstieae*, but *Dicymbe* and *Phyllocarpus* in the *Sclerolobieae*, at the same time stating that *Dicymbe* and *Dicymbopsis* are nearly related. The

[1]) The figures indicate the number of species that were investigated
— = negative reaction
G = galactomannan present in the seeds
S = starch present in the seeds.

problematical genus *Thylacanthus* would be nearly related to *Dicymbe*. DUCKE too seems to have felt the difficulties caused by the recognition of the three tribes!

As since BENTHAM's days a large number of species have been discovered, and as many species have meanwhile become better known, it seems that a new classification, which is urgently needed, should be attempted. In my opinion, LÉONARD's methods of experimental taxonomy offer a good perspective. Perhaps the occurrence of amyloid in the seeds may serve as a useful character.

The following genera of Table II lack amyloid:

a) *Batesia, Campsiandra, Melanoxylon, Poeppigia, Sclerolobium, Tachigalia.*
b) *Gossweilerodendron, Hardwickia, Kingiodendron, Oxystigma.*
c) *Pterogyne.*
d) *Colophospermum.*
e) *Stahlia.*

The genera referred to under a) constitute the bulk of the *Sclerolobieae* in the sense of BENTHAM: only *Tachigalia* belongs, according to BENTHAM, to the *Amherstieae*. However, DWYER (1954a) adduced convincing arguments for a relationship between *Tachigalia* and *Sclerolobium*.

The genera referred to under b) are nearly related; to this group we should refer *Prioria* (LÉONARD, 1957) and perhaps *Stahlia*. This group of genera might, as well as group a), occupy a special place in the combined tribe *Amherstieae-Cynometreae-Sclerolobieae*. Group b) would occupy also a special place in the *Cynometreae* of LÉONARD.

The genus *Pterogyne* does not seem to belong to this tribe s.l., but to the tribe *Dalbergieae* of the *Leguminosae-Papilionatae*; the anatomy of the wood led COZZO (1951) to this conclusion. The deviating number of chromosomes also points to the desirability of a reclassification of *Pterogyne* (TURNER and FEARING, 1959).

The genus *Colophospermum* occupies an isolated place in the tribe s.l., although it undoubtedly belongs to the latter (LÉONARD, 1957). The number of its chromosomes (36) is exceptional, the most common number in the tribe being 24 (TURNER and FEARING, 1959).

Summarizing the results obtained by means of the amyloid-reaction on members of the *Leguminosae-Caesalpinioideae*, it may be said that amyloid-containing genera are found exclusively in the tribes *Cynometreae-Amherstieae-Sclerolobieae*. In these tribes two groups of genera with amyloid-lacking species occur, while the majority of the genera consists of amyloid-containing species.

Amyloid is a major constituent of the amyloid-containing seeds of the *Leguminosae-Caesalpinioideae*. In *Tamarindus indica* seeds, for instance, it occurs in the cotyledons in quantities of about 60 %. It is not surprising, therefore, that already several of the earlier authors came to the conclusion that amyloid is a reserve substance (FRANK, 1866; GODFRIN, 1884; HEINRICHER, 1888; REISS, 1889; RIEDEL, 1897).

The correlation existing between the occurrence of amyloid in species of the *Leguminosae-Caesalpinioideae* and the position of these species in the taxonomic system is therefore an example of a correlation

existing between physiological and morphological characteristics in the sub-family.

It is interesting to note that similar correlations between physiological and morphological characters are to be found in some other tribes of the sub-family. Galactomannan occurs very generally and in large quantities in the well-developed endosperm of species belonging to the tribes *Cassieae* and *Eucaesalpinieae*, while the seeds of the *Swartzieae* contain starch as a reserve polysaccharide.

The conclusions with regard to the occurrence of amyloid in the *Leguminosae-Caesalpinioideae* cannot be final since only part of the species were studied. Of the about 730 species belonging to the tribes *Amherstieae-Cynometreae-Sclerolobieae* about 110 species have been investigated; the investigated species belong to about 57 out of the 92 genera. Of the roughly 700 species belonging to the other tribes 76 species were investigated, belonging to 45 out of the 62 genera.

B. Other plant families

In other plant families amyloid was found in cotyledons or endosperm of the species listed in Table v. About 2500 species belonging to 208 different families of the *Spermatophyta*, were tested. In 16 dicotyledonous families amyloid-containing species were found, but none in the *Monocotyledones*, of which 25 families were studied.

TABLE V

Amyloid-containing species in taxa other than *Leguminosae-Calsalpinioideae*

Acanthaceae
Anisacanthus virgularis Nees
wrightii Nees
Beloperone californica Benth.
Drejerella guttata (T.S. Brandegee) Brem.
Hypoëstes phyllostachya Bak.
sanguinolenta Hook.
Peristrophe bicalyculata Nees
Ruspolia seticalyx Milne-Redhead
Schaueria calycotricha Nees
Schwabea ciliaris Nees
Annonaceae
Annona cherimolia Mill.
montana Macfad.
muricata L.
reticulata L.
squamosa L.
Artobotrys brachypetalus Benth.
uncinatus Merrill.
Asimina triloba Dun.
Cananga odorata Hook. f. et Th.
Hexalobus glabrescens Hutch. et Dalz.
Mezzettia parviflora Becc.
Monodora crispata Engl.
myristica Dun.
tenuifolia Benth.
Polyalthia littoralis Boerl.
suberosa B. et H.
Popowia caffra Hook. f.
Rollinia emarginata Schlecht.
Stelechocarpus schefferi Boerl.
Unona discolor Vahl
Uvaria macrophylla Roxb. var. microcarpa Finet et Gagn.
rufa Blume
Xylopia aethiopica A. Rich.
mendoncae Exell.
villosa Chipp.
Balsaminaceae
Impatiens balsamina L.
Limnanthaceae
Limnanthes douglasii R. Br.
Linaceae
Linum bienne Mill.
grandiflorum Desf.
perenne L.
usitatissimum L.
Melianthaceae
Melianthus major L.
minor L.
Myrsinaceae
Ardisia acuminata Willd.
crenata Sims
humilis Vahl
polycephala Wall.
wallichii D.C.

TABLE V (*continued*)

Maesa alnifolia Harv.
argentea Wall.
indica (Roxb.) Wall.
lanceolata Forsk.
perlarius (Lour.) Merr.
Myrsine africana L.
Rapanea neriifolia Mez.
urvillei Mez.
Papilionaceae
Goodia lotifolia Salisb.
Mucuna urens Medic
Pedaliaceae
Ceratotheca triloba E. Mey.
Harpagophytum peglerae Stapf
Josephinia imperatricis Vent.
Sesamum indicum L.
orientale Sieber
radiatum Schum.
Primulaceae
Anagallis arvensis L.
foemina Mill.
Androsace maxima L.
Ardisiandra wettsteinii J. Wagner
Asterolinum stellatum (L.) Link et Hoffm.
Centunculus minimus L.
Coris monspeliensis L.
Cortusa matthioli L.
Cyclamen cilicium Boiss. et Heldz.
europaeum L.
graecum Link.
neapolitanum Tenore
persicum Mill.
hybr.
Dodecatheon meadia L.
Douglasia laevigata A. Gray
vitaliana Rox.
Glaux maritima L.
Hottonia palustris L.
Lysimachia lichiagensis Forrest.
mauritiana Lam.
punctata L.
thyrsiflora L.
Primula officinalis Jacq.
sinensis Lindl.
uralensis Fisch.
Samolus valerandi L.
Soldanella alpina L.
carpatica Vierh.
montana Willd.
Steironema ciliatum (L.) Raf.
Trientalis europaea L.
Ranunculaceae
Paeonia albiflora Pall.
anomala L.
beresowskii Komarov.
corallina Retz.
coriacea Boiss.
decora Anders.
delavayi Franch.
lusitanica Mill.
lutea Del.
macrophylla (Alb.) Lomak
mlokosewitschi Lomak
mollis Anders.
moutan Sims.
obovata Maxim.
officinalis L.
paradoxa Anders.
peregrina Mill.
potanini Komarov.
tenuifolia L.
triternata Pall.
trollioides Stapf.
veitchii Lynch.
wittmanniana Hartw.
woodwardii Cox.
Sapindaceae
Cardiospermum halicacabum L.
hirsutum Willd.
Sapotaceae
Achras sapota L.
Dipholis montana Griseb.
salicifolia A.D.C.
Illipe spec.
Mimusops balata Miq.
elengi L.
hexandra Roxb.
Omphalocarpum ahia A. Chev.
anocentrum Pierre
Sideroxylon australe Benth.
foetidissimum Jacq.
inerme L.
quadriloculare Pierre
Theophrastaceae
Jacquinia pungens A. Gray
Thunbergiaceae
Thunbergia alata Boj. ex Sims subspec.
alata Brem.
subspec. reticulata Brem.
fragrans Roxb.
Tropaeolaceae
Tropaeolum majus L.
minus L.
peltophorum Benth.
peregrinum L.
speciosum Poepp. et Endl.

a. *Acanthaceae* (sensu Brem.)

Among sixty-two species belonging to 49 different genera 10 amyloid-containing species were found; the latter species appeared

to occur in the tribe *Justicieae* (sensu Brem.). The amyloid occurs in the cell-walls of the cotyledons.

b. *Annonaceae*

The cell-wall of the endosperm of all investigated *Annonaceae* (25 species) stained brownish-violet with the reagent. The reaction was judged to be positive in all cases.

c. *Balsaminaceae*

In the cotyledons of *Impatiens balsamina* amyloid was found; in six other *Impatiens* species and in *Hydrocera triflora* the reaction was negative.

d. *Leguminosae-Papilionatae*

Goodia lotifolia has amyloid in the cotyledons in contrast to *Goodia pubescens* in which it is absent.

Mucuna urens was the only one out of four *Mucuna* species which proved to contain amyloid. Of 104 genera of *Papilionatae* 126 species have been tested.

e. *Limnanthaceae*

Of this small family the only species investigated appeared to have amyloid-containing cotyledons.

f. Linaceae

The endosperm cell-walls of 3 out of 11 *Linum* species gave a positive reaction. The amyloid-containing species belong to the section *Eulinum*. Amyloid was not found in *Hugonia swynnertoni* nor in *Radiola linoides*.

g. *Melianthaceae*

Of this small family two *Melianthus* species were tested; the endosperm cell-walls of both species contain amyloid.

h. *Myrsinaceae*

All species investigated have amyloid-containing endosperm. The *Maesa* species, however, show only a slightly positive reaction.

i. *Pedaliaceae*

The cotyledon cell-walls of the six investigated species give a very slightly positive reaction.

j. *Primulaceae*

The endosperm cell-walls of all investigated species react positively.

k. *Ranunculaceae*

All the *Paeonia* species investigated have endosperm cell-walls with amyloid. The other *Ranunculaceae* that were tested (30 species belonging to 27 genera), have no amyloid.

l. *Sapindaceae*

Of 28 species (belonging to 18 genera) only *Cardiospermum hali-*

cacabum and, very slightly, *C. hirsutum* were found to have amyloid-containing cotyledon cell-walls.

m. *Sapotaceae*

Twenty-three species (belonging to 11 genera) were tested; 12 species (belonging to 6 genera) contain amyloid.

n. *Theophrastaceae*

One member of this small family was tested; the endosperm cell-walls contain amyloid.

o. *Thunbergiaceae* (sensu Brem.)

Three *Thunbergia* species were tested which appeared to have cotyledons with thickened cell-walls, containing amyloid.

p. *Tropaeolaceae*

All the investigated species have amyloid-containing cotyledon cell-walls.

In some instances a correlation exists between the taxonomic position and the occurrence of amyloid.

Of the families belonging to the order *Primulales* (*Primulaceae*, *Myrsinaceae* and *Theophrastaceae* the endosperm of all investigated species has cell-walls containing amyloid.

The families *Linaceae*, *Tropaeolaceae*, *Limnanthaceae*, *Balsaminaceae*, *Melianthaceae* and *Sapindaceae* are related to each other. According to Dalla Torre and Harms (1900–1907) they belong to the orders *Geraniales* and *Sapindales*.

The families *Acanthaceae*, *Thunbergiaceae* and *Pedaliaceae* are inter-related and belong to the order *Tubiflorae*.

In the *Ranunculaceae* only the genus *Paeonia* was found to have species with amyloid-containing seeds. On account of its morphological characters this genus occupies an isolated place in the family, and it has been proposed to exclude the genus from the *Ranunculaceae* (e.g. by Davesne, 1957).

The genus *Cardiospermum* was the only one of the *Sapindaceae* where amyloid-bearing species were encountered, but of the tribe *Paullinieae* to which *Cardiospermum* belongs, only one more species, viz. *Serjania clematidifolia*, was tested. Perhaps an investigation of more species of this tribe will reveal other amyloid-containing genera.

The amyloid-positive species of the *Sapotaceae* are spread over the subfamilies *Mimusopoideae* and *Chrysophylloideae*; in the *Madhucoideae* amyloid positive species were not found. In the *Chrysophylloideae* also a number of negative species were encountered.

As already stated, the seeds of all the investigated species of the *Annonaceae* give a brownish violet colour with the iodine reagent. A study of the structure of this cell-wall substance which has been classified among the amyloids, is under way.

In summarizing the results obtained with plant taxa other than the *Leguminosae-Caesalpinioideae* we may say that a number of plant taxa

seem to consist exclusively of amyloid-containing species (*Annonaceae*, *Primulaceae*, *Myrsinaceae* and perhaps *Theophrastaceae*, *Limnanthaceae*, *Tropaeolaceae*, *Melianthaceae*, *Pedaliaceae*, *Thunbergiaceae*), while in other families amyloid occurs more or less occasionally (*Linaceae*, *Balsaminaceae*, *Sapindaceae*, *Sapotaceae*, *Ranunculaceae*, *Acanthaceae*, *Leguminosae-Papilionatae*).

Plant species with seeds containing amyloid will certainly appear to be much more numerous than we know at present (the number is now about 230). Besides the possibility that more plant families will prove to comprise amyloid-containing species, the fact that of the species of the amyloid-containing families only a fraction has been tested, leaves but little doubt that still numerous amyloid-positive species will be discovered.

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SUMMARY

By the application of potassium triiodide to dry sections of seeds amyloid was observed in the cell-walls either of the cotyledons or of the endosperm of the following plants:

1. All genera of the tribes *Cynometreae-Amherstieae-Sclerolobieae of the Leguminosae-Caesalpinioideae* with the exception of two groups of genera (Table II and III); in the other tribes of this subfamily no amyloid was found (Table IV).

2. All investigated species of the *Primulales*, the *Anonaceae*, *Limnanthaceae*, *Melianthaceae*, *Pedaliaceae Thunbergiaceae* and *Tropaeolaceae* (Table V).

3. A number of species of *Balsaminaceae*, *Acanthaceae*, *Leguminosae-Papilionatae*, *Linaceae*, *Ranunculaceae*, *Sapindaceae* and *Sapotaceae* (Table V).

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DUAL EFFECT OF NIGHTBREAK LIGHT

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Abstract

Experiments with *Salvia occidentalis*, a short-day plant, showed that red nightbreak light which normally is effective in causing a long-day effect, can under certain conditions antagonize the long-day effect of a supplemental light period, thus causing a short-day effect. This short-day effect of nightbreak light disappears when the length of the nightbreak period is increased or when it is followed by an irradiation with far red (= near infra red).

Introduction

In a previous paper (Meijer, 1959) it has been demonstrated with several photoperiodically sensitive plants that at least two different photoperiodic reactions are involved in a long-day effect, viz. a main-light-period reaction and a nightbreak reaction. The nightbreak reaction, especially sensitive to red light, depends on a main-light-period reaction which is specially induced by far red or blue radiation. Further the effectiveness of far red and blue radiation in the main-light-period reaction is antagonized by red light. Such an antagonism between different spectral regions also exists in the case of the nightbreak reaction, the effect of red radiation being nullified by a following irradiation with far red or blue.

In this paper experiments are described which show that red nightbreak light not always causes a long-day effect and can even reverse the long-day effect of a long-day treatment under certain conditions.

Methods and material

In these experiments *Salvia occidentalis*, an obligate short-day plant, was used; the arrangement for irradiation has been described before (Meijer, 1957). The main light period was given with blue light as it was found that this light quality is more effective than white light in causing a long-day effect. For the sake of convenience the duration of the main light period and of the supplemental light period is given in number of hours, the duration of a night interruption in number of minutes followed by an apostrophe. The temperature was kept constant at 22° C. The duration of the treatment was 28 days after which the plants were transferred to long-day conditions in a glasshouse.

Experimental results

With *Salvia* it was found (Meijer, 1959) that the ineffectiveness of supplemental red light of low intensity, in changing a short day into a long day, was not due to an inactivity of the supplemental light period, but to an antagonizing effect of the first part of the red-light period which followed immediately after the main light period.

The following experiment was carried out to investigate whether the same inhibition occurred with a nightbreak treatment. Groups of 4 plants each were exposed daily to a main light period of 8 hours of blue light. One group was kept in darkness during the remaining 16 hours (8 B). Other groups were irradiated:

a) supplementarily for another 4 hours with red (8 B 4 R) or blue light (8 B 4 B);
b) with 15 minutes of red nightbreak light (8 B–15′ R) during the dark period, 7½ hours before the main light period started again;
c) with a combination of the supplemental 4 hours of red or blue light and the 15 minutes of nightbreak light (8 B 4 R–15′ R and 8 B 4 B–15′ R).

A similar series of treatments was carried out but with a main light period of 10 hours of blue light per day instead of the 8 hours period of the foregoing series (10 B, 10 B 4 R, etc.).

The intensity of the blue light was 680 $\mu W/cm^2$ and of the red light 830 $\mu W/cm^2$. The results are given in Table I.

Table I

The photoperiodic effect of supplemental light and nightbreak light following after a main light period of 8 hours and 10 hours of blue light. Intensity of blue light 680 $\mu W/cm^2$ and of red light 830 $\mu W/cm^2$. Duration of the treatment 28 days. + = generative (short-day effect); — = vegetative (long-day effect). Observations after 54 days

Main light period in hours per day	Supplemental light period in hours per day	Nightbreak in minutes per day	Conditions of the growing points
8 B	—	—	+ + + +
8 B	—	15′ R	— — — —
8 B	4 R	—	+ + + +
8 B	4 R	15′ R	+ + + +
8 B	4 B	—	+ + + +
8 B	4 B	15′ R	— — — —
10 B	—	—	+ + + +
10 B	—	15′ R	— — — —
10 B	4 R	—	— — — —
10 B	4 R	15′ R	+ + + +
10 B	4 B	—	— — — —
10 B	4 B	15′ R	— — — —

In preliminary experiments it was found that an exposure to 12 hours of red or blue light per day caused a short-day effect. An irradiation of 14 hours of blue light caused a long-day effect; 14 hours of red light were not completely effective in this respect.

As can be seen in Table I a supplemental light treatment with

4 hours of red or blue light was only effective in obtaining a long-day effect when given after a main light period of 10 hours (10 B 4 R and 10 B 4 B), but not after 8 hours of light per day (8 B 4 R and 8 B 4 B).

As expected, a long-day effect could also be obtained with nightbreak light after a main light period of 8 or 10 hours of blue light (8 B–15′ R and 10 B–15′ R).

However, when the main light period was composed of 8 hours of blue + 4 hours of red light (which by itself induced a short-day effect), no long-day effect could be obtained by a nightbreak of 15 minutes (8 B 4 R–15′ R = short day).

Still more remarkable was the fact that the nightbreak light caused a short-day effect after a main light period of 10 hours of blue + 4 hours of red light, which by itself resulted in a long-day effect (10 B 4 R = long day, 10 B 4 R–15′ R = short day).

When the main light period consisted of 14 hours of blue light, no such activity of the nightbreak light could be observed (14 B = long day, 14 B–15′ R = long day).

In another experiment, the results of which are given in Table II, it was found that by increasing the length of the nightbreak period after a main light period of 10 B 4 R a long-day effect was obtained again. In this experiment the light intensities of blue and red light were 750 μW/cm² and 810 μW/cm², respectively.

Table II

The influence of the length of the nightbreak period on the photoperiodic effect of a supplemental light treatment. Intensity of blue light: 750 μW/cm² and of red light: 810 μW/cm². Duration of the treatment 28 days. + = generative (short-day effect); — = vegetative (long-day effect). Observations after 55 days.

Main light period in hours per day	Supplemental light period in hours per day	Nightbreak in minutes per day	Conditions of the growing points
10 B	—	—	+ + + +
10 B	4 R	—	— — — —
10 B	4 R	5′ R	+ + + +
10 B	4 R	10′ R	+ + + +
10 B	4 R	15′ R	+ + + —
10 B	4 R	30′ R	+ + — —
10 B	4 R	60′ R	+ — — —

As can be seen in Table III, the short-day effect of red nightbreak light (10 B 4 R–10′ R) was also annulled when it was followed by an irradiation with far red (10 B 4 R–10′ R 10′ FR).

Conclusion

It is clearly shown in this paper that it depends on the preceding light period whether nightbreak light causes a long-day effect. It is quite remarkable, however, that a nightbreak given after a special treatment which just causes a long-day effect even reverses this long-day effect. This short-day effect of a nightbreak disappears when its length is increased or when it is followed by far-red radiation.

TABLE III

The influence of far red on the effect of red nightbreak light. Intensity of blue light 680 $\mu W/cm^2$; of red light 870 $\mu W/cm^2$ and of far red 300 $\mu W/cm^2$. Duration of the treatment 28 days. + = generative (short-day effect); — = vegetative (long-day effect). Observations after 51 days.

Main light period in hours per day	Supplemental light period in hours per day	Nightbreak in minutes per day	Conditions of the growing points
10 B	—	—	+ + + +
10 B	—	10′ R	— — — —
10 B	—	10′ R 10′ FR	+ + + —
10 B	4 R	—	— — — —
10 B	4 R	10′ R	+ + + +
10 B	4 R	10′ R 10′ FR	— — — —

So it appears that under certain conditions the short-day plant *Salvia occidentalis* behaves like a long-day plant, nightbreak causing flower initiation. A special light treatment caused only vegetative growth (10 B 4 R); a nightbreak of 10 minutes of red light added to this main light period induced flowering (10 B 4 R–10′ R).

A comparable phenomenon has been reported by DE LINT (1959) for *Hyoscyamus niger*, a long-day plant. Under certain conditions flowering was obtained; an interruption of the dark period, however, caused vegetative development, i.e. a short-day effect.

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CHROMOSOME NUMBERS OF SOME EQUISETUM SPECIES FROM THE NETHERLANDS

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(*received November 18th, 1959*)

Equisetum Linn. is the sole surviving genus of a "microphyllous" group—the *Equisetineae*—the members of which formed a conspicuous element in the vegetation of the earth during the Carboniferous period. Prior to Manton's (1950) exhaustive work, exceedingly conflicting data were found in the literature with regard to the chromosome numbers of the various species. At present this genus is one of the few pteridophytic genera which have been thoroughly worked out cytologically. So far, out of a total of about 25 recognized species (at an earlier date Baker (1887) described only 20 species) 18 species (see Table II) have been studied (cf. Manton, 1950; Ninan, 1955 and Mehra et Bir, 1959). Significant enough is the fact that all these species have identical chromosome number (n = 108), and the question that faces us at present is how this high number ($n = 108$) has been arisen. For this reason a critical and extensive study of representatives of this group obtained from various geographic regions of the world is highly desirable. The present paper is a sequel to a previous communication from this laboratory and deals with cytological observations made on four species occurring in the Netherlands.

Material and Method

The Netherlands are very rich in "horsetails", and as many as 10 species are reported to occur in this country (cf. van Ooststroom, 1948). Cytological material of only four of the latter, *viz. E. arvense* L., *E. palustre* L., *E. fluviatile* L. (sub-genus *Equisetum*) and *E. trachyodon* A. Br. (subgenus *Hippochaete*) was available for study. The distribution data with regard to these species are based on van Ooststroom's (*loc. cit.*) account of the Pteridophytes in Flora Neerlandica I.

In all cases the young to medium-sized cones were fixed in 1:3 acetic alcohol and preserved in the latter. Table I gives exact sources of the cytological material.

The fixed material, along with pressed specimens from exactly the same localities, were kindly sent to me by Dr. K. U. Kramer. In the usual way aceto-carmine squashes were made at Amritsar in April, 1959, *i.e.* about nine to ten months after the fixation. The

[1]) The present study has been made possible through the kind help of Dr. K. U. Kramer (Utrecht), who supplied the fixed material as well as the herbarium specimens.

drawings have been made from temporary preparations, because many of the permanent proved to be distored. The degree of fertility of the spores was estimated after the spores had been stained with acetocarmine and had been mounted in 50 % glycerine.

TABLE I

Name of the species	Locality	Habitat	Date of Collection	Collector
E. arvense L.	De Bilt (Prov. Utrecht)	Grassy, sandy soil	22nd June, 1958	Dr. K. U. Kramer
E. palustre L.	Vechten, S. E. of Utrecht	Shallow boggy ditch, ca. 20 cm water	22nd Juni, 1958	Dr. K. U. Kramer
E. fluviatile L.	Veenendaal (Prov. Utrecht)	Swampy grassland	16th June, 1958	Dr. K. U. Kramer
E. trachyodon A.Br.	Near Asperen Neder-Betuwe (Prov. Gelderland)	Swampy spot, flooded in winter	13th May, 1958	Dr. J. C. Lindeman

OBSERVATIONS

E. arvense, *E. palustre* and *E. fluviatile* occur throughout the Netherlands, the last-mentioned species, however, is not common in the west and south-west. *E. arvense* is a weed of waste places and road sides, while the two other species prefer moist habitats.

All three species are generally distributed in the North Temperate and Arctic zones of both hemispheres, but *E. palustre* is rare in south Europe, and *E. fluviatile* flourishes only in ponds and lakes (cf. BAKER, *loc. cit.*). *E. arvense* is a polymorphic species, and from Michigan state (U.S.A.) alone six forms have been recognized (cf. BILLINGTON, 1952). *E. arvense* L. var. *boreale* (Bong) Ledeb. (= *E. arvense* L. ssp. *boreale* (Bong) Löve) is Arctic circumpolar in distribution.

As pointed out in an earlier communication (MEHRA et BIR, *loc. cit.*) the size and configuration of the bivalents is very variable. The chromosomes are slightly brittle and may break during squashing. The resulting fragments can easily be mistaken for small individual bivalents (cf. Fig. 2). Therefore, for exact counting, a large number of cells have been analysed in each case. In all three species, *E. arvense*, *E. fluviatile* and *E. palustre*, the haploid chromosome number is $n = 108$ (Figs. 1–2). In Fig. 2, which represents a spore mother-cell of *E. fluviatile*, in addition to the 108 bivalents two small, mechanically caused fragments are present. Such fragments usually lie near the respective bivalents and can easily be relegated to them. The spore mother-cell at prophase or diakinesis or even at metaphase usually shows 1–3 conspicuous nucleoli, one being large and generally vacuolated in the centre, while the remaining ones are small and almost equal in size. The latter are generally circular in outline.

The course of meiosis is perfectly normal, resulting in well developed spores. The mature spores contain numerous chloroplasts and fully

developed elaters. There are no traces of "plasmodial residue" intermixed with the spores.

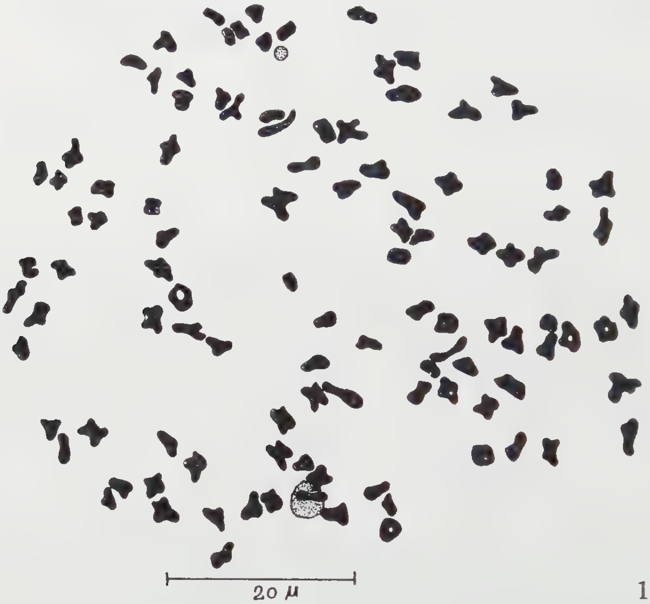

Fig. 1. A spore mother-cell of *E. arvense* showing n = 108.

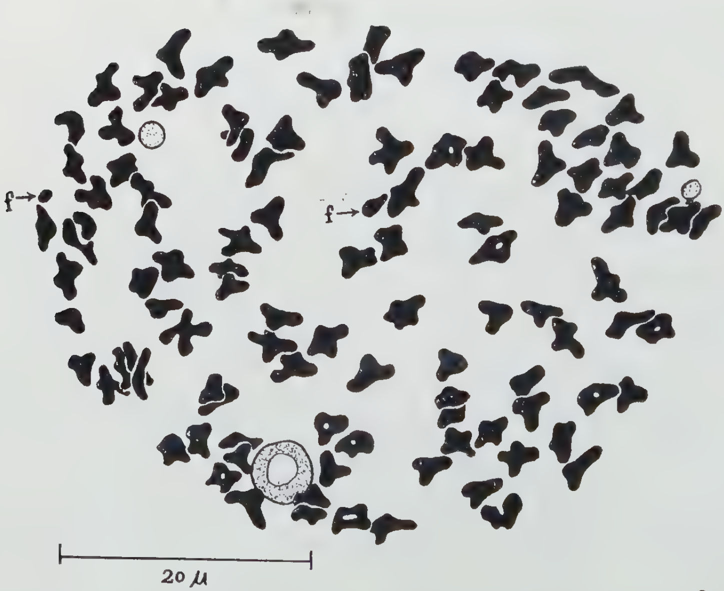

Fig. 2. Diakinesis in a spore mother-cell of *E. fluviatile* showing 108 bivalents. and 2 mechanically caused fragments marked "f".

E. trachyodon A. Br.

This species has a comparatively restricted distribution; according to BAKER (*loc. cit.*) it is found in France, Germany, Ireland and Scotland. In the Netherlands it has recently been discovered, and is extremely rare (VAN OOSTSTROOM en REICHGELT, 1960). It was collected by Dr. J. C. Lindeman from a single station near Asperen,

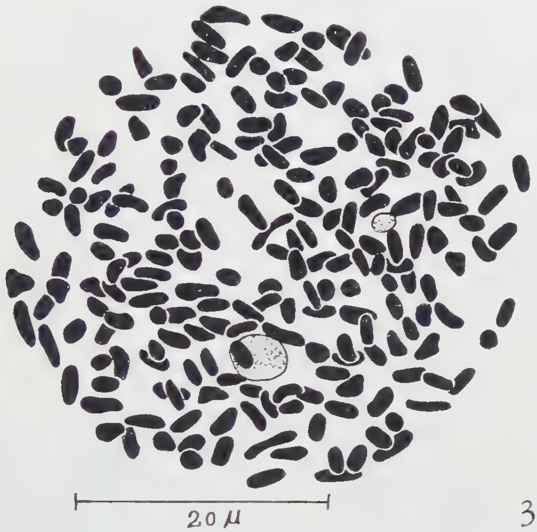

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Fig. 3. A spore mother-cell of *E. trachyodon* showing 216 univalents.

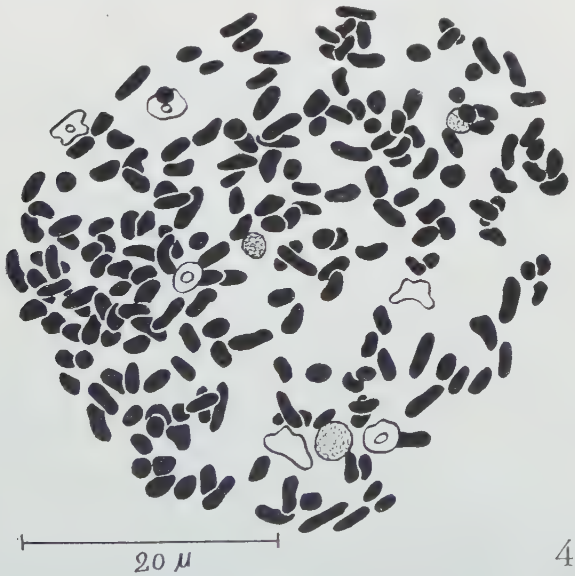

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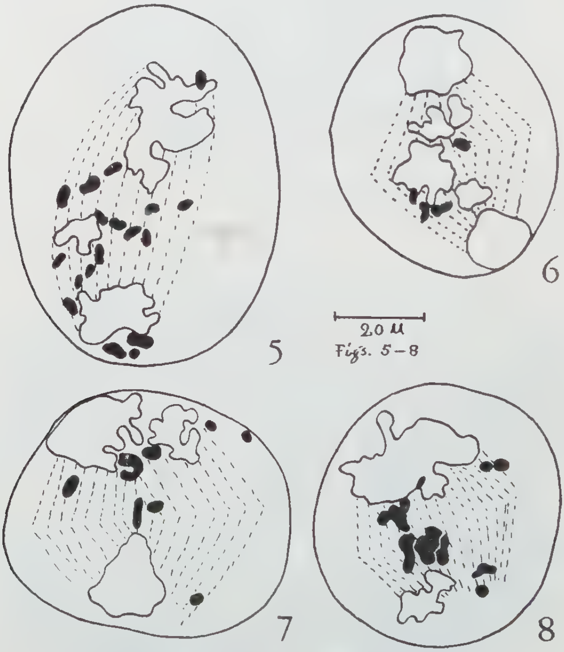
Fig. 4. Another cell of *E. trachyodon* with 204 univalents and 6 bivalents, thus giving 2n = 216.

Neder-Betuwe (see Table I). It flourishes in swampy places which are usually flooded in winter.

Cytologically this species is rather interesting. Meiosis is exceedingly irregular, at diakinesis in the majority of the spore mother-cells there is a complete failure of pairing, and 216 univalents have been counted (Fig. 3). However, one spore mother-cell (Fig. 4) showed 6 bivalents and 204 univalents, giving $2n = 216$. The number of nucleoli present at this stage is 2–3, one being large and one or two small. This species has previously been studied from Ireland (at almost the same latitude as the Netherlands) by MANTON (*loc. cit.*) and she too has reported an almost complete absence of pairing. Numerous similar cases of total asynapsis are known from ferns.

The metaphase and anaphase of the heterotypic division are highly abnormal. The chromosomes are irregularly scattered on the spindle, and their distribution to the two poles is very unequal (Figs. 5–8). In most of such cells the spindle is bipolar and straight. However, in a few cells this bipolar spindle may be so strongly curved that the



Figs. 5–8. Irregular meiosis in *E. trachyodon*; 5–6. anaphase I showing chromosomes irregularly scattered on the spindle; 7–8. showing irregular distribution of chromatin material to the poles.

two poles come very near to each other (Fig. 9). The organization of such bow-shaped spindles has been studied in *Impatiens pallida* Nutt. (SMITH, 1935), *Beta* (PRYWER, 1931), and in *Trichomanes insigne* v. d. B. forma β (MEHRA et SINGH, 1957). Quite often in some mother cells a tripolar spindle is formed, and "spindle fibers" are developed

between the three poles (Fig. 10). OKABE (1929) has also reported tripolar spindles in spore mother-cells of *Psilotum nudum*. But in certain cells the connecting fibers are not developed between each pair of nuclei. Therefore, while the chromosomes are distributed on two sides, no trace of them is evident on the third (Fig. 11). Spore mother-cells with this type of spindle are of common occurrence. Meiosis occasionally does not proceed up to or beyond anaphase I, and so within a mother cell 1–2 irregular nuclei may be formed. The daughter cells ultimately round off, resulting in the formation of monads or dyads. The interkinetic nuclei within the mother cells are rarely circular in outline.

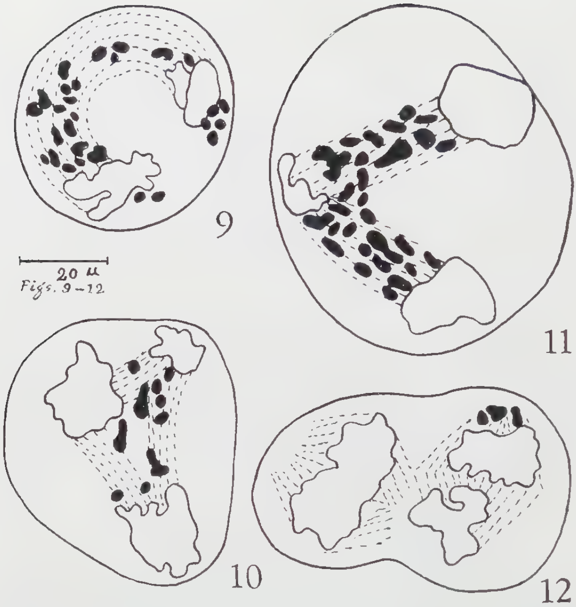
The second homeotypic division takes place very rarely, and when it occurs, it is also highly abnormal. Metaphase II and anaphase II are irregular (Fig. 12) and ultimately as a result of either failure of or incomplete cytokinesis the resultant spores are of irregular shape and variable size. The amount of chromatine material is also variable. In mother cells where both divisions fail, a large nucleus is found, and the mother cell finally rounds off as a monad spore. Ultimately these spores abort (Fig. 13). Such spores have only two coats, exosporium and endosporium being developed from the spore-protoplast. The "middle layer" and outer perenium which ultimately constitutes the elaters are not developed. Consequently the nucleated "plasmodial fluid" which normally contributes towards the development of "middle layer" and elaters (cf. BEER, 1909) is not fully utilized, and bits of "plasmodial residue" can be seen intermixed with sterile spores. In normally reproducing species of *Equisetum* no such "residue" is mixed up with the spores.

DISCUSSION

E. trachyodon is cytologically abnormal, and frequently in the spore mother-cells tripolar spindles are formed. This phenomenon may possibly be related to an abnormal constitution of the species, as in all normal forms characteristic bipolar spindles have been observed.

As already pointed out, at diakinesis there is almost complete failure of pairing in this species. The probable causes of asynapsis and the consequential disturbances (such as a curved spindle, scattering of chromosomes at metaphase and their unequal distribution) may be due to three causes; (1) to the effect of extreme changes of temperature to which the individual may be exposed, and which may upset the normal metabolic processes, (2) to gene mutations, or (3) to hybridity.

The abnormalities in *E. trachyodon* cannot be due either to the temperature effect or to gene mutations, because the Irish populations of this species investigated by MANTON (*loc. cit.*) showed similar irregularities as the material from the Netherlands. Therefore, a hybrid origin remains the most probable explanation of the abnormalities. The pairing at diakinesis is practically nil in the majority of the cells. This suggests that parents with different genomic constitution



Figs. 9–12. Irregular meiosis in *E. trachyodon*; 9. shows a bow-shaped spindle with some chromosomes scattered all over it; 10. tripolar spindle with fibres developed between the three poles; 11. tripolar spindle showing the absence of "spindle fibres" on one side; 12. homeotypic division, metaphase II and anaphase II.

Fig. 13. Abnormal and abortive spores of *E. trachyodon*, fragments of "plasmodial residue" are seen intermixed with these.

may have been involved in the origin of this species, and that the chromosomal complements contributed by the two parents may have been totally non-homologous.

The hybrid nature of *E. trachyodon* has been suspected since long, and BAKER (*loc. cit.*) spoke of this species as "midway between *E. hyemale* and *E. variegatum*". According to MANTON (*loc. cit.*), a relationship of *E. trachyodon* with the south European *E. ramosissimum* has sometimes been suggested. In the opinion of the author the latter species differs morphologically so strongly from *E. trachyodon* that it cannot be regarded as one of the parents. From the distribution map given by MANTON (*loc. cit.* p. 227) it is clear that *E. trachyodon* is well distributed in Ireland, though in the rest of Europe it is rare. So far, this species has been cytologically investigated from two different sources, Ireland (MANTON, *loc. cit.*) and the Netherlands (present report), and from both the places the hybrid nature of the species is evident.

The question that arises next, is how this hybrid has become so widely distributed. It cannot be distributed by spores, because of the sterility of the latter. Only two possibilities suggest themselves, viz. (1) either the hybrids have arisen independently in each locality, or (2) the distribution from one common source has taken place in a remote past by vegetative means, *i.e.* by means of rhizomes. A study of distributional data (cf. BAKER, loc. cit. and MANTON, *loc. cit.*) of *E. hiemale*[1]), *E. variegatum* and *E. trachyodon* shows that the areas of these species overlap. So it may have originated many times and at many places. It is also possible that once having arisen by hybridization it has propagated vegetatively through rhizomes which are known to be very hardy and resistant in the genus *Equisetum*.

Before discussing the evolutionary status of *Equisetum* in the light of cytological evidence, it would be proper to summarize the available data which are presented in Table II.

Löve (personal communication), who has worked out the somatic chromosome numbers of a large number of Arctic and N. American species of *Equisetum* (cf. Table II), has confirmed the earlier view of MANTON (*loc. cit.* p. 215–218) that there are some differences in the size of the chromosomes in the two sub-genera viz. *Equisetum* and *Hippochaete*. Originally the division of *Equisetum* into two sub-genera was proposed by MILDE (1867) because of the structure of the stomata, and since then this division has been followed. Recently ROTHMALER (1944) raised these quite distinct *Equisetum* sections to generic rank. The difference in relative chromosome size (meiotic and somatic) of two the sub-genera gives some support to ROTHMALER's (*loc. cit.*) proposal.

A perusal of Table II shows that thirteen species have been worked out from two or more geographically different regions and that all

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[1]) There is some difference in the spelling of the specific name. BAKER (loc. cit.) BILLINGTON (1952) and HAUKE (1958) write it as *hyemale*. The spellings used in this paper has been adopted from the "Index Londinensis" Vol. III, 1930. Oxford.

TABLE II

Resume of chromosome numbers in *Equisetum*[1])

Name of the species	Source	Chromosome number n	Chromosome number 2n	Reproduction	Author
sub-genus Eu-equisetum:					
E. arvense L.	England	ca. 108	—	Normal	Manton (1950)
	Netherlands	108	—	Normal	Present report
E. arvense L.	Iceland	—	216	—	Löve (unpublished)
ssp. *boreale* (Bong) Löve	N. America	—	216	—	Löve (unpublished)
E. maximum Lam.	England	ca. 108	—	Normal	Manton (1950)
(= *E. telmateia* Ehrh.)	N. America	—	216	—	Löve (unpublished)
E. sylvaticum L.	England	ca. 108	—	Normal	Manton (1950)
	Iceland	—	216	—	Löve (unpublished)
	N. America	—	216	—	Löve (unpublished)
E. pratense Ehrh.	Hort.	ca. 108	—	Normal	Manton (1950)
	Iceland	—	216	—	Löve (unpublished)
	N. America	—	216	—	Löve (unpublished)
E. palustre L.	England	ca. 108	—	Normal	Manton (1950)
	Netherlands	108	—	Normal	Present report
	Iceland	—	216	—	Löve (unpublished)
	N. America	—	216	—	Löve (unpublished)
E. limosum L.	England	ca. 108	—	Normal	Manton (1950)
(= *E. fluviatile* L.)	Netherlands	108	—	Normal	Present report
	Iceland	—	216	—	Löve (unpublished)
	N. America	—	216	—	Löve (unpublished)
E. diffusum D. Don	Darjeeling, E. Himalayas	108	—	Normal	Mehra et Bir (1959)
	Mussoorie, W. Himalayas	108	—	Normal	Mehra et Bir (1959)
E. litorale Kuhn	Ireland	Irregular meiosis	—	Sterile hybrid	Manton (1950)
sub-genus Hippochaete:					
E. robustum A. Br.	Botanic Garden	ca. 108	—	Normal	Manton (1950)
E. ramosissimum Desf.	Itly	ca. 108	—	Normal	Manton (1950)
E. ramosissimum Desf. var. *altissimum* A. Br.	Darjeeling, E. Himalayas	108	—	Normal	Mehra et Bir (1959)
	Mussoorie, W. Himalayas	108	—	Normal	Mehra et Bir (1959)

[1]) The information about chromosome numbers of Icelandic and N. American species of the genus referred to here, has very kindly been supplied by Dr. Askel Löve (Montreal). However, nothing is known about the nature of spores and reproduction.

TABLE II *(continued)*

Name of the species	Source	Chromosome number n	2n	Repro-duction	Author
E. hiemale L.[1])	England	ca. 108	—	Normal	Manton (1950)
	Iceland	—	216	—	Löve (unpublished)
	N. America	—	216	—	Löve (unpublished)
E. scirpoides[1]) Michx.	Norway	ca. 108	—	Normal	Manton (1950)
	N. America	—	216	—	Löve (unpublished)
E. variegatum[1]) Schleich	British Isles	ca. 108	—	Normal	Manton (1950)
	Iceland	—	216	—	Löve (unpublished)
	N. America	—	216	—	Löve (unpublished)
E. trachyodon A. Br.	Ireland	Irregular meiosis	—	Sterile hybrid	Manton (1950)
	Netherlands	216I	—	Sterile hybrid	Present report
E. Moorei Newm.	Ireland	Irregular meiosis	—	Sterile hybrid	Manton (1950)
E. debile Roxb.	South India	108	—	Normal	Ninan (1955)
	North India	108	—	Normal	Mehra et Bir (1959)
E. laevigatum A. Br.	Michigan, N. America	Irregular meiosis	—	Sterile hybrid	Hauke (1958)

[1]) Askel Löve (personal communication) following ROTHMALER (1944) prefers the names *Hippochaete hiemalis*, *H. scirpoides* and *H. variegata* respectively for these species.

possess identical chromosome numbers. Among 17 species worked out so far, 4 hybrids have been detected. This is a fairly high number, and it speaks for the evolutionary activity still present in this very ancient group. The presence of high chromosome numbers (which is decidedly not primitive) is probably an indication of high antiquity. *Equisetum* is surviving from the Carboniferous period to the present day with unbroken continuity, and it is therefore a relic of the past. It seems possible that during this process all the members with lower chromosome numbers have become extinct. From what basic number the present number may have been evolved, and what is the grade of ploidy in the genus *Equisetum*, are two important questions that still remain unanswered.

SUMMARY

The cytology of four Netherlandic species of *Equisetum* has been worked out. *E. arvense*, *E. fluviatile* and *E. palustre* are normal and show n = 108, while *E. trachyodon* is almost a totally asynaptic species with 2n = 216. The results are in complete agreement with those obtained by Manton (1950) for these species. In *E. trachyodon* the course of meiosis has been critically examined, and it is concluded that this is a hybrid species as has already been suspected by earlier workers. The present-day evolutionary status of the genus has been discussed in the light of cumulative cytological evidence.

ACKNOWLEDGEMENTS

The author expresses his extreme sense of gratitude to Prof. P. N. Mehra for encouragement and kind guidance, to Dr. K. U. Kramer and Dr. J. C. Lindeman (Utrecht) for fixation of the material, and to Dr. Askell Löve (Montreal) for helpful suggestions and also for supplying cytological information about N. American and Icelandic species of *Equisetum*. He is also thankful to Mr. B. Khanna for help with the drawings.

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SENECIO CONGESTUS (R.Br.) DC. IN THE LAKE YSSEL POLDERS

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(*received February 2nd, 1960*)

1. Introduction

An arctic paramorph of the species was treated as *Senecio congestus* (R.Br.) DC. in 1837 and this appeared to be the correct name (Fernald, 1945). Therefore the name *Senecio tubicaulis* Mansf., proposed in 1940, must be rejected. The binominal *Senecio palustris* (L.) Hook. (1834), frequently used for this species, is excluded by *Senecio palustris* Velloso, published for a S. American taxon in 1827.

As to the polymorphy of the European material no exact data are available. Ruprecht (1846) separated the plants from the arctic part of Europe under the name *S. arcticus*, but it is questionable whether this material merits specific rank. According to Fernald (1945), who distinguished three varieties of *S. congestus* in N. America, the var. *palustris* (L.) Fern. might be identical with the Eurasian material; this paramorph being treated in some Floras as var. *palustris* (L.) Hyl. But with regard to this question a closer examination is desirable.

This treatise refers to a very small part of its area of distribution, where the species shows a slight variability in morphological characteristics only and so *S. congestus* is not subdivided.

S. congestus is a boreal species, which occurs on both sides of the Atlantic. According to Hegi (1929) its area of distribution comprises: N. Russia, S. Sweden, Esthonia, Latvia, Lithuania, Denmark, Poland, Czechoslovakia, Hungary, Germany, the Netherlands, Belgium (very rare; Goffart, 1945), N. France (probably extinct; Fournier, 1946), British Isles (extinct; Clapham, Tutin and Warburg, 1952) and Siberia. Fernald (1950) records the species from the Northern part of N. America.

Van Ooststroom (1956) states that the species is locally common in the Netherlands, especially to the North of the line Zuid-Beveland, Dordrecht, Nijmegen; not being known from Zuid-Limburg.

After the enclosure of the Zuiderzee (since 1932 called Lake Yssel), soon followed by desalinization, *S. congestus* established itself on the bare and muddy shores, which came into being as a result of the disappearance of the tidal movement (Feekes, 1936, 1941). In all probability, its diaspores were wind-borne from the adjacent peaty habitats, e.g. the Twiskeland (Westhoff, 1943), Noordwest-Overijssel (De Leeuw, 1929; Van Soest, 1937; Feekes and Bakker, 1954) and the vicinity of Kampen (Van Soest, 1933) (Fig. 1).

In the saline Wieringermeer, after the emergence of the land from

Fig. 1. Reclamations in the Lake Yssel with adjacent areas.

the water in 1930, a *S. congestus* aspect occurred only locally, viz. in fresh seepage regions (FEEKES, 1936). On the other hand, in the two polders (North-Easternpolder and Eastern Flevoland), which had been drained dry after the enclosure of the Zuiderzee in 1942 and 1957 respectively, the species established itself temporarily on a large scale. In the fresh or faintly brackish North-Easternpolder a *S. congestus* aspect occupied about 1200–1400 ha during the 1943–1944 season (FEEKES and BAKKER, 1954). In the nearly fresh Eastern Flevoland this aspect was found on at least 10.000 ha from August 1958 till July 1959. The first establishments in those polders mainly arose from seed, disseminated from small sources along the coast of the Lake Yssel.

After the optimum years 1943–1944 and 1958–1959, in the North-Easternpolder and Eastern Flevoland respectively, *S. congestus* decreased rapidly. At present the species occurs only sporadically and

with a low vitality in the North-Easternpolder, whereas it covers 1000 ha at most in Eastern Flevoland. Probably the species will decrease still more in the latter polder during the following years.

This phenomenon was also observed shortly after the reclamation of lakes in the Netherlands during preceding centuries. In this connection it may be of interest to quote GEVERS VAN ENDEGEEST (1861) after the reclamation of the Haarlemmermeer in 1852: 'From the mud millions of plants grew up after recession of the water, rushes and reed and a large number of willows, and in the muddiest soils the Marsh fleawort, the well-known waterplant of our reclamations, which disappears without trace after one or two years. From afar this plant with its yellow flowers gave an impression as if the drained soils had turned into rape fields; the wind-borne seeds were disseminated as far as the surrounding towns'.

Consequently, in two of the three polders in the Lake Yssel and in the Haarlemmermeer the establishment of *S. congestus* took place rapidly on a large scale, but was followed by a strong decline after some years. In addition, in Eastern Flevoland it was observed that species with vegetative propagation often started to take possession of the open spaces immediately after the *S. congestus* vegetations had died off, especially *Phragmites communis*, but also *Scirpus maritimus* and *Typha latifolia*. Thus, in some months a *S. congestus* aspect changed into an aspect of *Phragmites*, *Scirpus maritimus*, *Typha latifolia* or into a mixture.

In some instances, however, species with vegetative propagation did not occur and after *S. congestus* had died off, development of a thin vegetation took place during the first years only. Locally, on the other hand, the perished vegetation of *S. congestus* was succeeded by a new generation of the same species, which became equally vigorous.

In order to find an explanation of these phenomena several ecological characteristics of the species were studied. The majority of the field trials have been made in Eastern Flevoland. The results will be dealt with in this paper.

2. LIFE FORMS

S. congestus is a hapaxanthous species. In the Netherlands the majority of the population are winter annuals. The seed of the winter annuals germinates in the June-August period; the plants survive the winter with leaf-rosettes (mainly one rosette per plant), which are lost before flowering in May and June. The dissemination chiefly takes place in June, whereas the plants die in July. The dead stems may largely cover the soil surface during several months.

In the Lake Yssel polders only the winter annuals play an important part in the vegetation. Hence this study refers mainly to this life-form.

Moreover, in June and July flowering summer annuals may arise from seed that has survived the winter. Their vitality is considerably smaller than that of the first winter annual generation, as had been

observed by VALLIN (1925) in S. Sweden. Besides, in autumn flowering summer annuals occur, viz. a small part of the plants grown from seeds, which have germinated immediately after dissemination in June, may flower in September and October. Their first generation usually shows the same vitality as the first generation of the winter annual life-form.

According to HEGI (1929) perennial plants of *S. congestus* are found too, but in the Lake Yssel region they have never been observed. In this connection, it should be noted that sometimes plants with more than one leaf-rosette may occur. In this case, stems and flower-stalks arise exceptionally from rosettes before the winter, whereas other rosettes of the same plants do not produce them before the next spring.

It seems possible that these differences in life-form are connected with phenomena of vernalization and photoperiodism, but this has not been studied.

As to the structure of the root system, *S. congestus* belongs to the type with adventitious roots. The primary root and the lateral roots are slightly developed and they are lost in the juvenile stage. Some weeks after germination adventitious roots, arising from the base of the rosettes, start growth and they increase considerably in length and number before the winter.

With regard to the factor water, the plant should be considered as a telmatophyt (IVERSEN, 1936), as it prefers unaerated, wet and muddy soils, where the species is capable of taking root by means of a strongly developed system of air-canals in leaves, stems and roots. The adventitious roots are surrounded by a thick layer of aerenchyma tissue (Fig. 2).

Pollination may be achieved in different ways. The heads have about 21 female ray-florets and 80–140 hermaphrodite disc-florets, rich in honey and pollen. Hence they are much visited by insects (Coleoptera, Diptera, Hymenoptera and Lepidoptera). But pollination is also achieved in the absence of insects. For the tips of the style-arms of the disc-florets bear small brushes, collecting pollen shed into the anther-tubes during emergence of the styles. After the style-arms have curled downwards the stigmatic surfaces come into contact with pollen on the brushes.

In Eastern Flevoland, where on the Knardijk (Fig. 1) about 800 bee-hives had been placed along the *S. congestus* vegetations in May 1959, some information was obtained about the fruit-setting of entomophilous and automophilous pollinated heads. It appeared that the bees visited the flower-heads mainly within a distance of 500–600 m from the Knardijk. At a distance of 1000 m the author sporadically observed bees, and other pollinating insects as well.

In this area thirty heads with mature fruits (in this paper generally called seed) have been collected at random at a distance of respectively 50, 1000 and 2000 m from the bee-hives on the Knardijk. Of each sample the germination was determined, as is shown in Table I. In view of the fact that the non-germinating seed did not exhibit red

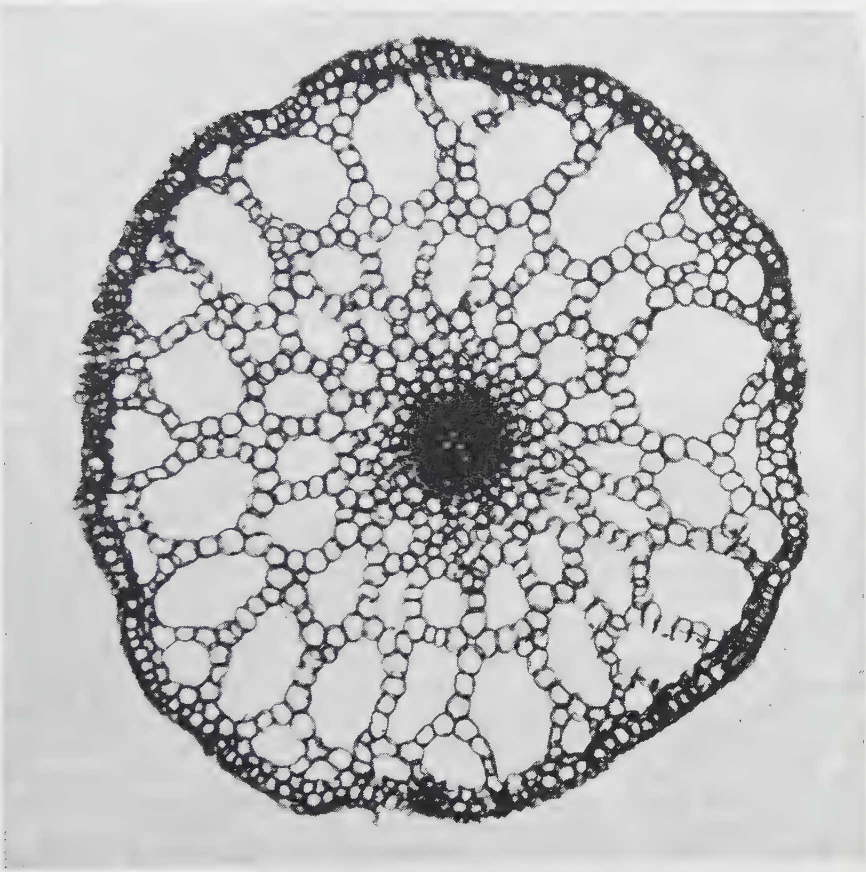

Fig. 2. Adventitious root of *S. congestus* in transverse section, showing the aërenchyma layer (75 ×).

colouring after treatment with sodiumbiselinite, the germination was taken as a measure of the fruit-setting.

It follows from Table I that the extent to which pollinating insects occurred and the degree of fruit-setting did not correlate. Although an explanation of this phenomenon cannot be given, it is clearly shown that fruit-setting may take place on rather a high level in a

TABLE I

Viability of *S. congestus* seed in, respectively, presence (50 m) and nearly complete absence (1000 and 2000 m) of pollinating insects. In brackets the limits of variation.

	Mean germination % at a distance from the Knardijk of: 50 m	1000 m	2000 m
Series 1	38 (11–80)	57 (25–85)	29 (6–63)
Series 2	67 (20–95)	77 (39–98)	77 (35–97)

nearly complete absence of pollinating insects, in other words under pioneer conditions.

As to dissemination and influence of mineral nitrogen and NaCl an ample discussion will follow under 6, 7 and 8.

3. Habitats according to literature

Regarding the habitats in Germany Hegi (1929) makes the following statement: 'Trupp- oder herdenweise, vielfach nur vorübergehend (mitunter auch verschleppt), in frischen Torfstichen, auf unberasten Torfhügeln, in Torfgräben, bisweilen auf sumpfigen und schwach berasten Moorwiesen, Hochmooren, an Teich- und Seeufern, auf abgelassenen Teichen. Nur in der Ebene und im Hügellande. Die Pflanze zählt zu den sehr bezeichnenden Moorpflanzen, wenngleich sie bisweilen auch massenhaft auf Faulschlamm an Seeufern auftritt, begleitet von Equisetum, Heleocharis, Scirpus paluster und S. Tabernaemontani, Acorus Calamus, Rumex Hydrolapathum, Stellaria palustris, Nasturtium palustre, Cicuta virosa, Galium palustre usw. Am liebsten stellt sie sich aber auf frischen Torfstichen, Torfabfallhaufen, an Torfgräben usw. ein. Sie ist an solchen Orten vielfach mit Carex pseudocyperus, C. Oederi, C. cyperoides, Cladium Mariscus, Cirsium rivulare, Nasturtium amphibium, Oenanthe aquatica vereinigt. Auch mit Pirola uniflora kann sie zusammentreffen. Gegen stärkeren Rasenschluss ist sie infolge ihrer fast stets zu beobachtenden Kurzlebigkeit empfindlich und daher an vielen Orten unbeständig'.

According to Van Ooststroom (1956) in the Netherlands the species may be found in marshes and peat-bogs, but is sometimes numerous on newly reclaimed fresh soils and on newly cut peat. On the other hand, in the opinion of Feekes (1941) it prefers a peaty, wet and faintly brackish environment.

Vallin (1925) records *S. congestus* from the desalinized landwardsides of seaweed-banks, washed ashore on the island of Väderö in S. Sweden. Here a nitrophilous vegetation occurred, in which *Ranunculus sceleratus* showed the greatest frequency.

Nordhagen (1940) found that the vegetations of the desalinized landwardsides of the seaweed-banks along the Norwegian coast and on the island of Väderö were highly comparable, but *S. congestus* did not occur in the Norwegian localities. In the opinion of Nordhagen these vegetations belong to the nitrophilous *Bidentetum tripartiti* Koch. It is questionable, however, if *S. congestus* may be considered as a faithful species of this association as a result of inconstancy ('meteorische Pflanze').

On the other hand, in Germany Roll (1939) regards *S. congestus* as a faithful species of the *Bidentetum tripartiti*, together with *Bidens tripartitus*, *Ranunculus sceleratus*, *Polygonum hydropiper* and *Alopecurus geniculatus*. He points to the low competetive power and to the nitrophilous character of the association.

Sissingh (Westhoff, Dijk, Passchier and Sissingh, 1946) classes *S. congestus* with the faithful species of the *Rumicetum maritimi*, a pioneer

association on embanked or dredged up soils. The other faithful species recorded are *Rumex maritimus* and *Ranunculus sceleratus*.

The name *Rumicetum maritimi* has been changed into *Ranunculetum scelerati* by TÜXEN (1950), with the following faithful species: *Ranunculus sceleratus*, *Rumex maritimus*, *R. palustris* and presumably *S. congestus*. This association is found: 'Auf feuchten bis nassen, an Stickstoff und Nährstoffen reichen offenen Böden am Rande von Viehtränken, Dorfteichen, auf der Sohle abgelassener Fischteiche und auf Abwasser Versickerungsflächen (Rieselfeldern), auch in Flachmoor-Torfstichen'.

From the literature mentioned above the conclusion may be drawn, that *S. congestus* is a pioneer of wet and bare soils. According to HEGI (1929) the rapid decline may be the result of the low competitive power, but this holds good as well for the species which usually grow together with *S. congestus*.

The species shows its optimum development on soils rich in mineral nitrogen, e.g. on seaweed-banks and dredged up soils, along ponds etc., where nitrophilous summer annuals frequently occur. Moreover, *S. congestus* is often found together with *Ranunculus sceleratus*, an extreme hydrophilous species.

Apparently there has been some controversy in literature as to the question whether *S. congestus* shows a NaCl-tolerance or not.

4. OBSERVATIONS ON THE VEGETATION-CYCLE OF *S. congestus* IN EASTERN FLEVOLAND

On five sample plots, each 100 m² (10 × 10 m) in extent, the vegetation-cycle was studied in Eastern Flevoland during the years 1957–1959 (Table II). The plants on these plots took root in silty clay loam, emerging from the water in the spring of 1957. During germination and seedling growth, which took place in the summer of 1957, the soil surface was wet and bare. Shallow pools even covered the plots 3 and 4 till the autumn of 1959.

On all the plots the aspect was formed by *S. congestus* rosettes during the autumn and winter of 1957. Nevertheless, plot 5 showed a much thinner cover than plots 1–4. And so on plots 1–4 a very dense vegetation of flowering *S. congestus* occurred in June 1958, whereas at that time on plot 5 an open vegetation was met with, in which *S. congestus* and *Ranunculus sceleratus* predominated. This difference may be attributed to the fact that in the summer of 1957 plots 1–4 were 0.5–1.5 km remote from the nearest fruiting sources of *S. congestus*. But this distance amounted to 7 km for plot 5. It follows that the thin cover of the latter plot should be the result of the low accessibility (HEIMANS, 1940) to *S. congestus*. This was clearly shown in the summer of 1957 by means of a sowing experiment, performed at a distance of about 0.3 km from plot 5. Here a nearly continuous vegetation of rosette-plants was found in the autumn of the same year.

In June 1958 the total cover had increased on all plots in comparison with the situation in September 1957. On plots 1–4 the increase was mainly due to the growth of the *S. congestus* plants. However, the greatest increase took place on plot 5, but here vigorous *Ranunculus*

sceleratus plants played an important part too. This might be the result of the rather open vegetation in the latter case, where competition was still of slight influence.

The total cover on all plots proved to have decreased in September 1958, but not in the same measure, whilst plants of the new *S. congestus* generation were either entirely absent or of low vitality. It was found that this decrease of the total cover depends on the degree in which species with vegetative propagation are present. The occurrence of the latter appeared to be connected with edaphic factors and accessibility, as will be discussed below.

Twenty nine seedlings of *Phragmites communis*, sown in May 1957, grew on plot 1 of Table II in September 1957. Furthermore six seedlings of *Scirpus maritimus* were found, arising from seed deposited at the bottom by the water of the Lake Yssel, prior to reclamation. In all probability they were disseminated from the *Scirpus* vegetations along the coast, about 1 km away. Owing to the heavy vegetation of the first *S. congestus* generation the vegetative propagation of *Phragmites* and *Scirpus maritimus* was hampered. But they could increase considerably by means of vegetative propagation after *S. congestus* had died off, because the soil surface was thinly overgrown and still wet at that time. It appeared that in September 1958 the *S. congestus* aspect of June 1958 had changed into a *Phragmites* aspect and the vegetation was nearly continuous in June 1959.

On plot 2, about 0.4 km away from plot 1, nearly the same edaphic factors prevailed as on plot 1. Nevertheless, on account of the scarce establishment of *Phragmites* (two plants), the vegetation on plot 2 remained thinner than on plot 1. This might be explained by the fact that the vegetative propagation of *Phragmites* takes place more rapidly than that of *Scirpus maritimus*. The sparse establishment of *Phragmites* might be the result of the chance occurrence that not much seed had been deposited on plot 2.

On plots 3 and 4 the total cover decreased considerably after the first *S. congestus* generation had died off, because species with vegetative propagation were sparse or did not occur at all. The absence of *Phragmites* was due to an environment unfavourable to the establishment from seed (seepage pools), sown in 1957. Meanwhile *Phragmites* penetrated into plot 3 by means of vegetative propagation from a source along the dyke. The small scale establishment of *Scirpus maritimus* may be connected with the sporadic supply of diaspores. For the seed sources of this species were about 15 km away from these plots.

In view of the fact that in the summer of 1958 diaspores of *S. congestus* were deposited in excess on the plots 3 and 4, while the soil surface was wet and thinly covered with vegetation, the scarce establishment of the second generation could not be the result of low accessibility, drought or competition. An ample discussion of this phenomenon will follow under 8.

On plot 5 the species with vegetative propagation increased too, but rather slowly owing to the small number.

TABLE II

Vegetation-cycle of *S. congestus* on five sample plots (10 × 10 m in extent) in Eastern Flevoland after its emergence from the water in the spring of 1957. The frequently occurring species are recorded only. Italicized figures = fruiting plants.

Nr.		Soil surface mostly wet, 3 and 4 usually with shallow pools									
		September '57		June '58		September '58		June '59		September '59	
1.	Total cover	85 %		95 %		80 %		95 %			
		Cover	Stem height	Cover	Stem height	Cover	Stem height	Cover	Stem height	Cover	Stem height
			cm	%	cm	%	cm	%	cm		
	Ranunculus sceleratus	< 5 %	< 5	< 5	*34– 56*	< 5	< 5	5	*18– 37*	Brought under cultivation	
	Senecio congestus	75 %	31–42	80	*122–153*	—	—	—	—		
	Phragmites communis	29 ×	53–84	10	128–147	60	*165–198*	80	155–183		
	Scirpus maritimus	6 ×	26–42	< 5	39– 61	10	*82–103*	10	47– 66		
2.	Total cover	70 %		85 %		60 %		85 %			
	Ranunculus sceleratus	< 5 %	< 5	5	*39– 51*	10	< 5	< 5	*31– 46*	Brought under cultivation	
	Senecio congestus	65 %	25–46	70	*119–161*	5	< 13	10	*41– 84*		
	Phragmites communis	2 ×	42–60	< 5	111–140	15	*171–192*	35	140–176		
	Scirpus maritimus	13 ×	14–35	10	41– 73	25	61– 97	35	53– 83		
3.	Total cover	90 %		95 %		< 5 %		35 %		55 %	cm
	Ranunculus sceleratus	< 5 %	< 5	<5	*22– 43*	—	—	15	*28– 47*	5	*21– 47*
	Senecio congestus	85 %	35–48	90	*135–184*	< 5	< 7	< 5	*21– 53*	5	< 14
	Phragmites communis	—	—	—	—	—	—	< 5	22– 46	20	58– 77
	Catabrosa aquatica	< 5 %	< 12	< 5	*24– 36*	—	—	15	*16– 28*	25	< 11
4.	Total cover	85 %		95 %		10 %		20 %		55 %	
	Ranunculus sceleratus	< 5 %	< 7	< 5	*18– 41*	—	—	5	*16– 43*	5	*16– 39*
	Senecio congestus	80 %	28–44	90	*116–173*	< 5	< 8	< 5	*29– 66*	15	< 12
	Scirpus maritimus	1 ×	22–27	< 5	25– 43	5	*41– 69*	10	43– 65	15	*53– 91*
	Catabrosa aquatica	—	—	—	—	< 5	< 9	5	14	20	< 15
5.	Total cover	15 %		50 %		35 %		75 %		90 %	
	Ranunculus sceleratus	< 5 %	< 8	25	*27– 96*	< 5	< 5	25	*38– 57*	< 5	< 8
	Senecio congestus	10 %	23–39	20	*118–166*	< 5	< 9	< 5	*87–121*	—	—
	Phragmites communis	1 ×	31	< 5	78– 91	5	*128–151*	10	86–111	30	*143–191*
	Scirpus maritimus	5 ×	13–26	5	31– 64	20	*62– 91*	35	41– 83	55	*88–106*

In addition, a controversy with the data of Table II had been observed locally in the autumn of 1959. In some seepage pools in Eastern Flevoland vegetations of the second *S. congestus* generation appeared, with the same vitality as the first generation, at least in the rosette-stage. This phenomenon took place in pools which dried out during the extreme drought of June and July. Although the dry weather continued, the soil surface of the pools became wet again in August. In all probability the evaporation decreased at that time as a result of the shortening of the days, so that the seepage-water level rose. Under 8 an explanation of this phenomenon will be discussed.

5. Vitality

It is by no means simple setting up criteria of vitality valid for species with different life-forms. For this reason the criteria discussed below should be considered only an approach to the problem. The criteria are: seed output, dry matter production, stem height and number of flower stalks at the stem base. The figures recorded only refer to the winter annuals.

In order to obtain information about the seed output, ten plants of the first and second generations had been collected at random in June 1959. In this material the number of heads per plant and seeds per head as well as the percentage viable seed per head were determined. Finally the number of plants per 100 m² was counted. On the basis of these figures it was possible to calculate the mean output of viable seed per 100 m² (Table III). It can be seen from Table III that the seed output of the second generation had decreased in a high degree compared with the first generation.

TABLE III

Seed output of the first and second *S. congestus* generations in Eastern Flevoland (June, 1959). In brackets the limits of variation.

	Mean number:		Mean % viable seed per head	Per 100 m² mean number of:	
	heads per plant	seed per head [1]		plants [2]	viable seed
1st generation	978 (517–1731)	120 (81–161)	55 (23–81)	263 (111–474)	16 976 124
2nd generation	115 (43–223)	111 (71–152)	61 (42–74)	97 (0–223)	758 540

[1]) seed of 10 heads per plant counted.
[2]) plants of 10 × 100 m² counted.

Moreover, it was observed that plants of *S. congestus*, growing on bare and muddy soils without inter- and intraspecific competition, may produce 150.000–200.000 viable seeds. In other words, even after the establishment of a single plant a considerable increase might be achieved in the next year.

As to the dry matter production of *S. congestus* no data have been

recorded in literature. In Eastern Flevoland the production of the second generation mostly remained considerably behind that of the first one. In Table IV these figures are compared with the dry matter production of vigorous *Phragmites*, Winter wheat and Lucerne, growing in Eastern Flevoland. It appeared that in this respect the first generation partly coincided with the reed and the two crops. So it is likely that the first generation reached a high vitality in Eastern Flevoland. That this should be true can be deduced from the data concerning the stem height and the number of flower stalks at the base of the stems, as will be explained below.

TABLE IV

Annual dry matter production of some natural vegetations and agricultural crops in Eastern Flevoland.

Species	Dry matter production of the aerial parts in kg per ha
Senecio congestus, 1st generation, 1959 . . .	9000–11.000
Senecio congestus, 2nd generation, 1959 . . .	2000– 3.000
Phragmites communis, 3rd year, 1959	8000–13.000
Winter wheat, 1959	8000–14.000
Lucerne, 3–4 cuts, 1959	8000–14.000

First, in the majority of the W. European Floras it is stated that the maximum stem height amounts to 1 m (FOURNIER, 1946; CLAPHAM, TUTIN and WARBURG, 1952; VAN OOSTSTROOM, 1956). HEGI (1929) records: 'bis über 100 cm hohe Pflanze'. In S. Sweden on seaweed-banks VALLIN (1925) observed plants up to a height of 1.50 m. On the other hand, according to FERNALD (1950) the species does not exceed 0.60 m in N. America. From Table II it follows that the first *S. congestus* generation on the five plots reached a height of more than 1.50 m. FEEKES and BAKKER (1954), too, described from the North-Easternpolder first vegetations up to a man's height. In addition, the mean height of fifty *S. congestus* plants of the first generation, collected at random in June 1959, amounted to 1.54 m, with a variation of 1.29–1.93 m, whereas the second generation had a mean height of 0.71 m, with a variation of 0.38–1.09 m.

Secondly, according to HEGI (1929) the stems of *S. congestus* are only branched in the upper parts. But in the Lake Yssel polders the first generation even bears flower stalks at the base of the stems, e.g. these fifty plants: meanly 7 with a variation of 0–13. In the second generation this figure was 1 with a variation of 0–3.

All the figures recorded refer to vegetations on heavy soils. Nevertheless on wet and muddy, sandy soils the first generation may also exhibit the high vitality described.

6. DISSEMINATION

From a morphological point of view the *S. congestus* diaspores, formed by achenes and pappi, are well 'adapted' to anemochorous dissemination (Fig. 3). The more so as their weight is low, viz. about

700 mg per 1000 diaspores. And the fall velocity in calm air proved to be about 16 cm per sec. It should be noted, however, that these diaspores might be disseminated by water and animals too.

S. congestus diaspores are beaten out of the heads by rain. And in this case they land on the earth around the mother plants, because as a result of the high humidity opening of the pappi is prevented.

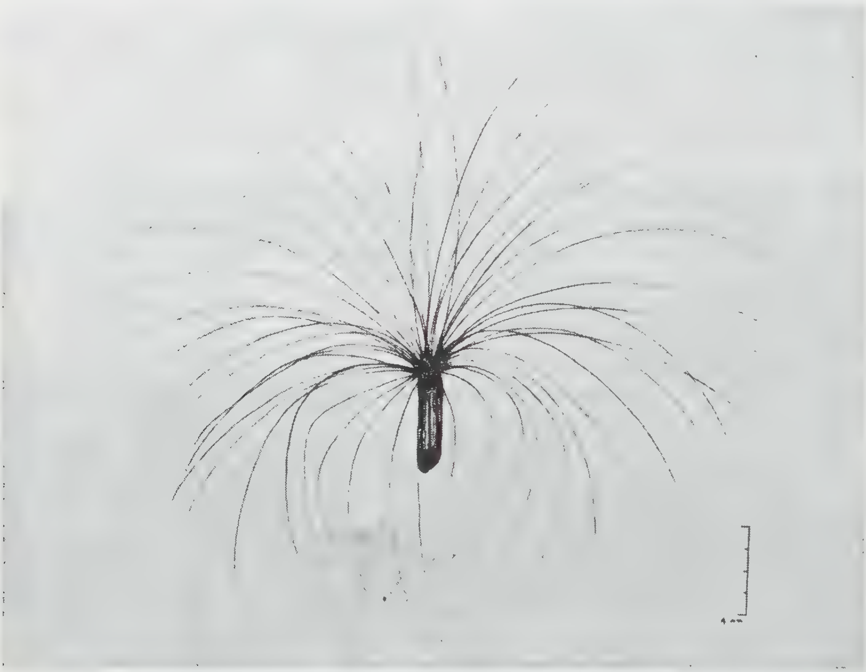

Fig. 3. Diaspore of *S. congestus*.

Dry weather favours air transport. Yet they are partly deposited in the neighbourhood of the mother plants too, especially in heavy vegetations on a large scale. But long-distance transport by wind and convection currents appears to be possible as well.

We possess very few exact data on the flying-distance of diaspores (MÜLLER, 1955). In this connection the most reliable observations have been made by FEEKES (1936) concerning the migration of *Aster tripolium* into the newly reclaimed Wieringermeer. From a source in the border area *Aster* migrated in three years and in three laps into the whole Wieringermeer area, 20.000 ha in extent, by way of anemochorous dissemination. This means that many diaspores must have covered a distance of 6–8 km. But according to BOUWMAN (1936) large numbers of diaspores were also observed in Schagen, a town 10 km from the Wieringermeer (Fig. 1).

In Eastern Flevoland in 1957 there was an opportunity to obtain

some information about the flying-distance of *S. congestus* diaspores. On a clay dump, 0.5 ha in extent and 0.3 km from the Eastern dyke of the new polder, a fruiting *S. congestus* field was found in June 1957. It appeared that this vegetation yielded 750 million viable seeds. At that time the wind blew from the East on several days, resulting in an intensive air transport of diaspores from the clay dump to the virgin soil in the new polder. Here, in the autumn of 1957, 2–7 km to the West of the source, the estimated number of plants amounted to 5 million. This estimate was based on countings of plants on fifty plots, each 100 m^2 in extent, which had been chosen at random. It follows that at least 0.65 % of the viable seed covered a flying-distance of 2–7 km. Still more to the West its establishment was prevented owing to the occurrence of rather deep water in June and July 1957.

More valuable data concerning the flying-distance of *S. congestus* diaspores were collected in 1959. As has been stated in the introduction a fruiting vegetation of the species covered at least 10.000 ha of Eastern Flevoland in June and July of that year. Doubtlessly this must be considered as an exceptionally extended source of dissemination. In that period dry winds blew nearly every day, mainly from the N.E., E, and S.W., while convection phenomena occurred frequently.

From this source countless numbers of diaspores were disseminated nearly all over the Netherlands and even over the German frontier (Fig. 4). Clouds of diaspores have been recorded as far as 90 km on days when the wind blew from the direction of Eastern Flevoland, e.g. in Amsterdam, The Hague, Rotterdam, Groningen, Nijmegen. As can be seen from Fig. 4 diaspores have even been collected at a distance of about 200 km, e.g. in Hanover and Cologne. It seems possible that they too orriginated from Eastern Flevoland, as will be pointed out below.

The present author followed a cloud of diaspores by car in a North-eastern direction over a distance of 90 km on June 27th. The density in which the diaspores had been deposited on the soil surface appeared to fluctuate considerably. Especially along obstacles, e.g. forests, houses and dams, the density proved to be relatively high, up to a maximum of 12 viable seeds per m^2. On level country the number mostly varied from 0–2 per m^2. But in many instances a correlation between the density of the deposited diaspores and the nature of the ground apparently did not exist. Yet, with growing distance from the source the ratio of viable and non-viable seed usually decreased, in all probability owing to the lower weight of the latter. But it proved impossible to make an estimate of the percentage of viable seed covering a certain distance, on account of the irregular distribution on the soil surface. For the same reason the percentage remaining behind in Eastern Flevoland could not be estimated.

Undoubtedly the clouds of diaspores have come from Eastern Flevoland. For in the remaining part of the Netherlands and in the adjacent countries *S. congestus* occurred only locally at that time and

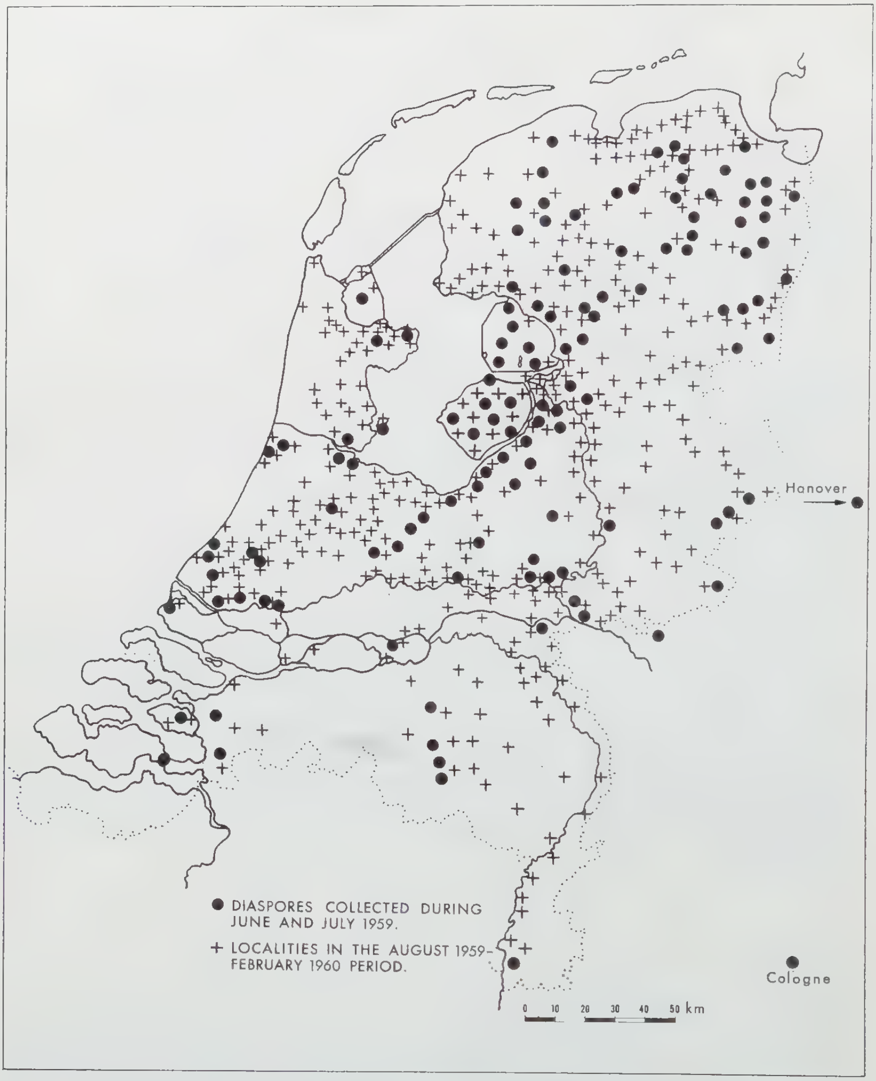

Fig. 4. Data on dissemination and establishment of *S. congestus* in the Netherlands.

in small sources, which would not have been capable of producing these large numbers of diaspores.

The dissemination from Eastern Flevoland in 1959 was followed by a large-scale establishment in the greater part of the Netherlands (Fig. 4). The local scarcity of the species, as recorded on the map of Fig. 4, may be caused by adverse conditions for its growth. But the direction of the wind during dissemination may have played a part as well. A study of this phenomenon is in progress.

In view of the fact that before August 1959 the plant grew sparsely or was wanting in the South and East of our country, occurrence in these regions should be paid special attention to. For on the basis of these data, it is likely that a part of the diaspores flew 90–160 km. It is due to this that the supposed origin of the diaspores collected in Hanover and Cologne gains in probability.

It is noteworthy that not only the large-scale anemochorous dissemination, but also the dry weather conditions must be responsible for the establishment all over the country. As a result of the drought, the water level of ditches, ponds, pools, rivers etc. fell during the summer of 1959, by which favourable habitats came into being nearly everywhere (see 7 and 8).

The question arises whether or not hydatochorous dissemination may be important for the species concerned. Laboratory tests on water with smooth and faintly waving surfaces showed that *S. congestus* diaspores remained floating for five days at most. In view of the fact that dissemination mainly takes place in June and July, the majority of the floating seed germinates within some days, on account of the high water temperature (see 7). If the seedlings are not washed ashore in an environment favourable to growth, they are lost after 1–2 weeks. It is evident now that at this time of the year hydatochorous dissemination over short distances can only play a part.

After landing on the water surface, seed of plants flowering in autumn does not, as a rule, germinate the same season owing to the low temperature. But partly, it is still capable of germination the next spring (see 7). Hence only the small seed output of the autumn plants (see 2) can be considered with regard to hydatochorous dissemination over long-distances and so hydatochorous dissemination cannot be as effective as anemochorous. All the more so as wind transport may follow the shortest way to a certain point, whereas in many instances well suited habitats cannot be reached by way of water unless by devious ways.

Now we shall have to discuss epizoöchorous dissemination. For the weak and short living achenes of the species endozoöchorous dissemination can be left out of consideration. Although not observed, epizoöchorous dissemination might be expected for *S. congestus*, in view of the fact that thousands of migrating ducks, geese and waders feed on the new soils.

In my opinion, however, epizoöchorous dissemination over long-distances is not likely to have been significant with pioneers in the Lake Yssel region. *Limosella aquatica* and *Cyperus fuscus*, which in the border area occur exclusively at the mouth of the river Yssel, have only been found in the North-Easternpolder and in Eastern Flevoland at a distance of at most 10 km from this source. In addition, *Scirpus americanus*, *Leersia oryzoides*, *Juncus obtusiflorus*, *Scirpus acicularis*, *Glyceria declinata* a.o. grew rather commonly in the border area along Eastern Flevoland. But they established themselves in the new polder at a distance of at most 3 km from the nearest sources. Between these areas and the polder many waterfowl flew to and fro. If long-distance

dissemination by birds were of significance in this case, the species should occur in the other parts of the polder as well. The more so as it was learned from sowing experiments that the absence of these species could not be attributed to edaphical factors. In all probability the species above mentioned were mainly disseminated by the water of the Lake Yssel before the dykes had been closed.

On the other hand, in the North-Easternpolder typical anemochorous species had been found, viz. *Hieracium caespitosum*, *Gnaphalium luteo-album* and *Epilobium lanceolatum*. Here they grew 20, 50 and 100 km respectively, from the nearest habitats outside the polders (Bakker and Van der Zweep, 1956). This may emphasise the significance of anemochorous dissemination over long-distances. Originally it was thought that *Carex extensa* had penetrated into the North-Easternpolder by means of endozoöchorous seed supply, e.g. from the Wadden Islands (Bakker and Van der Zweep, 1956). But a closer examination of the herbaria has shown that the species must formerly have been overlooked in the Zuiderzee region.

7. Influence of the environment on germination and seedling development

In this part, only the environmental factors which proved to be of major importance for germination and seedling development, will be discussed.

As to the ripening of the seed, it appeared that in the flower-head the seed is already capable of germinating to a maximum at the time the involucres are coloured brown; it does not exhibit any form of dormancy. This means that in an environment favourable to germination all the viable seed is able to germinate within a week. In this case, the embryos of the non-germinating seed do not show any red colouring after treatment with sodiumbiselinite. In other words, it is non-viable.

On the strength of field experience, it was thought that under natural conditions all the seed of the winter annual loses viability within three months. But in wet places it germinates immediately, even under adverse conditions for further growth, e.g. under heavy vegetation. On the contrary, it looked as if the seed of the summer annual, flowering in autumn, maintains its viability till the next spring.

In order to obtain information about this problem germination tests in the laboratory have been made. It appeared that viability decreased considerably after two months storage at about 21° C in combination with a high (59 %) or a rather high (23 %) moisture content of the seed. In the latter case, viability was nearly lost after 7 months' storage, whereas in the first case viable seed did not occur at all (nrs. 4 and 6 in Table v). On the other hand, at low temperature (8° C) the rate of germination proved to be independent of the moisture content of the seed for at least 7 months (nrs. 1, 3 and 5 in Table v). At about 21° C and a low moisture content (10 %) viability did

TABLE V

Influence of different modes of storage on the viability of *S. congestus* seed. In brackets the limits of variation.

Nr.	Mean germination %, immediately after harvest	Modes of storage		Mean germination % after:		
		tempera-ture	moisture content in %	2 months	7 months	14 months
1.	96 (94–100)	8° C	10	89 (80–97)	89 (84–95)	92 (90–95)
2.	96 (94–100)	± 21° C	10	84 (77–92)	78 (66–83)	81 (83–98)
3.	96 (94–100)	8° C	23	76 (71–81)	79 (72–81)	75 (60–86)
4.	96 (94–100)	± 21° C	23	63 (53–73)	7 (3–10)	0
5.	96 (94–100)	8° C	59	88 (72–89)	95 (90–98)	66 (59–79)
6.	96 (94–100)	± 21° C	59	20 (13–38)	0	0
7.	75 (73–76)	8–12° C	in soil, under water	—	14 (2–24)	0
8.	75 (73–76)	± 21° C	in soil, under water	—	30 (8–68)	8 (0—34)

not change after 14 months storage (nr. 2 in Table v). Storage took place in bottles hermetically closed with paraffin wax.

From the results of the laboratory tests the conclusion may be drawn that under conditions unfavourable to germination the seed loses viability in the summer in consequence of high temperature and moisture content. The moisture content of the disseminated seed amounts to at least 40 %. Independent of the moisture content seed of *S. congestus* may maintain viability at a high level during the winter as a result of low temperature.

Besides, the seed was kept under water (at a depth of 50 cm). It was learned that at 8–12° C and at about 21° C viable seed may still occur after 7–14 months' storage (Table v, nrs. 7 and 8). In this connection it seems possible that viable seed is to be found at the bottom of the Lake Yssel polders during recession of the water. This phenomenon was sporadically observed in Eastern Flevoland. Early flowering summer annuals with a low vitality arose from this seed.

With regard to the influence of 'depth of sowing', germination was also examined in laboratory tests. Aerated soil (vegetable mould) and unaerated mud from Eastern Flevoland were compared as germination substrata. The rate of germination was determined by counting the emerged seedlings. Table vi clearly shows that in both tests the maximum seedling production took place after sowing on the soil surface. Unlike when in the mud, however, seedlings still emerged from a depth of 2 cm out of vegetable mould. Undoubtedly this was

due to the considerable difference in aeration of the soil. Moreover, in vegetable mould large-scale germination proved to be possible, but the majority did not emerge, in all probability as a result of the mechanical resistance of the soil. In the unaerated mud the seed germinated only to a very small extent.

TABLE VI

Influence of 'depth of sowing' on the seedling emergence of *S. congestus*. In brackets the limits of variation.

	Mean % emerged seedlings at a 'depth of sowing' of:						
Germination substratum	0	0.5 cm	1 cm	1.5 cm	2 cm	2.5 cm	3 cm
Aerated vegetable mould	97 (94–100)	32 (26–40)	12 (3–19)	4 (1–5)	6 (2–8)	0	0
Unaerated mud	82 (79–83)	4 (0–8)	0	0	0	0	0

Referring to the results of Table VI, it is to be noted that under natural conditions the mainly anemochorous disseminated diaspores are usually deposited on the soil surface.

Nevertheless, germination may only proceed on soil surfaces covered with a thin film of water, like the young marine sediments in the Lake Yssel polders. Seedling development must take place under wet conditions, but in water of more than 2 cm depth penetration of the roots into the bottom usually does not succeed.

It appeared from field studies that a vigorous seedling growth is exclusively found on muddy substrata, e.g. on clay and peat, on newly deposited sand etc. It is noteworthy that in some instances *S. congestus* rosettes were even observed in ditches on thick layers of perishing *Lemna minor* and *L. gibba* or *Azolla filiculoides* (among others recorded by Mr. C. G. van Leeuwen in a letter of September 1959). On the other hand, on compact substrata, even under wet conditions, the seedling growth proved to be inhibited or quite impossible, e.g. on boulder clay, sand banks, gravel, marl. This might be connected with the occurrence of adventitious roots, adapted to life in unaerated soils. The thick and weak layer of aerenchyma tissue around these roots shows only a slight resistance to mechanical pressure, but in this direction closer examination is desirable.

As to the influence of temperature and intensity of light, it was learned that in full daylight maximum germination takes place at temperatures of 25–30° C. The seed may even germinate in the dark on a limited scale, but at lower temperatures (18–20° C) higher intensity of light is required. In weak light the seedlings perish after some time, e.g. under heavy vegetation. It follows that bare soils are favourable to germination and seedling development, because especially on the soil surface temperature and the intensity of the light may rise to high values during the growing season.

It was also observed that germination and seedling growth may be achieved in the pH-range 3.5–8, but at pH 3.5–5 the vitality is low.

TABLE VII

Influence of NaCl- and KNO_3-content of the substratum on germination of *S. congestus.* In brackets the limits of variation

Mean germination %:			
Series 1		Series 2	
Van der Crone's solution	40 (36–42)	Van der Crone's solution	40 (36–42)
Diluted sea-water, 1 g/l NaCl	18 (16–22)	Diluted sea-water, 1 g/l NaCl + 5 g/l KNO_3	55 (48–62)
Diluted sea-water, 3 g/l NaCl	15 (8–20)	Diluted sea-water, 3 g/l NaCl + 5 g/l KNO_3	31 (24–32)
Diluted sea-water, 5 g/l NaCl	12 (6–18)	Diluted sea-water, 5 g/l NaCl + 5 g/l KNO_3	26 (12–36)
Diluted sea-water, 10 g/l NaCl	12 (11–14)	Diluted sea-water, 10 g/l NaCl + 5 g/l KNO_3	7 (6–10)

The soils in the Lake Yssel polders, however, have mainly a pH within the suitable range (6.5–8).

Finally, the influence of NaCl on these life-stages has been studied in the laboratory, because in literature some controversy is met with respecting this question (see 3). Moreover, in Eastern Flevoland vigorous seedlings were found up to a NaCl-content of about 3 g per l soil moisture (C-figure), whereas in the brackish parts of the Netherlands *S. congestus* sporadically occurs, e.g. in the S.W. (Fig. 4).

Taking Van der Crone's solution as standard, the laboratory tests took place on diluted sea-water, artificially prepared and composed of two series, each containing solutions with 1, 3, 5 and 10 g NaCl per l respectively (Zijlstra, 1946). To the solutions of the second series 5 g KNO_3 per l was added. Table VII shows that on the NaCl-solutions of the first series germination did not reach the level of the Van der Crone's solution. But apparently under the influence of KNO_3, on 1 g NaCl of the second series germination surpassed the standard. It increased to a large degree on 3 and 5 g NaCl, in comparison with the first series. However, KNO_3 did not favour germination on the 10 g NaCl-solution. With regard to the NaCl-tolerance of the seedlings no reliable data were obtained. But on the basis of this test it seems possible that KNO_3 increases the NaCl-tolerance of this life-stage as well.

In view of the laboratory tests it was thought that the NaCl-tolerance might depend on the mineral nitrogen content in the soil. In support of this thesis is the fact that the young marine sediments in Eastern Flevoland are at first rich in mineral nitrogen (Table VIII).

TABLE VIII

Some edaphical factors of three *S. congestus* habitats after the first generation had died off compared with those of three adjacent bare fields (Eastern Flevoland, October, 1959).

		Died off first generation						Bare fields				
Depth	Nr.	[1]) NH_4^+	lutum in %	humus in %	NaCl[2]) in g/l	pH	←Distance→ about 2 m	[1]) NH_4^+	lutum in %	humus in %	NaCl[2]) in g/l	pH
0–20 cm	1	1.4	26.1	3.0	1.0	7.0		67.3	25.5	2.9	1.2	7.7
	2	1.6	7.5	1.8	0.9	7.2		20.0	9.8	1.8	1.1	7.6
	3	1.8	30.4	3.0	3.0	7.0		6.4	28.6	3.0	3.0	7.2
20–40 cm	1	1.8	41.2	3.2	1.9	7.3		92.4	35.2	3.4	2.2	7.7
	2	1.3	26.2	2.7	1.6	7.4		78.7	29.8	2.9	1.9	8.0
	3	6.9	33.7	3.6	3.3	7.3		66.2	28.6	3.1	3.0	7.5
40–60 cm	1	9.5	31.3	3.7	3.0	7.5		94.3	30.5	3.5	3.0	7.7
	2	8.4	26.4	3.2	2.4	7.4		82.1	27.6	3.3	2.4	8.0
	3	57.0	31.1	3.7	3.2	7.5		112.4	32.1	4.1	3.1	7.7
60–80 cm	1	88.4	33.4	4.6	3.6	7.5		132.6	33.7	4.7	3.7	7.5
	2	59.7	31.9	4.1	2.8	7.4		91.2	30.3	4.3	2.9	7.7
	3	118.9	34.5	4.8	3.6	7.5		124.3	31.5	5.2	3.7	7.6

[1]) mg mineral nitrogen per kg dry matter.
[2]) g NaCl per l soil moisture (C-figure).

8. Vitality decline

In this connection attention should be paid to the drying up of the soil surface. The first summer after recession of the water (1957) large areas of the heavy soils in Eastern Flevoland were covered with films of water necessary to germination of the species. But these films usually evaporated during dry periods in the second and third year. Yet dry periods did not prevent extensive germination in the summer of 1958, because as a result of rainfall the soil surface became wet again after some weeks of drought. In the very dry summer of 1959, however, the soil surface of large areas remained dry till the end of the growing season. For this reason, especially in the North-western part of Eastern Flevoland, the seed, deposited at the bottom, was not able to germinate after the first generation had died off in July 1959. On the other hand, germination and seedling growth took place in this area on four square metres, which were watered several times. In other words, with normal rainfall establishment might have been achieved.

It can be seen from Table II (nrs. 3 and 4) that also in permanently wet habitats a rapid vitality decline occurred. It seemed possible that the accumulation of dead stems of the first generation had caused a mulching effect. On this wet organic material, covering the soil surface to a maximum of 75 %, germination did not succeed, presumably owing to poisonous substances developed by anaerobic decomposition of the dead plants. In view of the fact that mostly the seedlings on the uncovered soils also showed a low vitality, even in case of immediate removal of the organic material after dying off, this phenomenon could not be the main cause of the vitality decline. Neither could seedling competition as a result of the high density be the general explanation, because in habitats thinly covered with seedlings of the second generation the vitality usually was low as well.

It is evident now that the rapid vitality decline should be attributed to changes in the edaphon, brought about under the influence of the first generation. As *S. congestus* mainly occurs together with nitrophilous pioneers (see 3 and 4), it was thought that this might be due to exhaustion of the mineral nitrogen by the first generation and by microbes and denitrification. It should be noted that the mineral nitrogen of unaerated and wet sediments in the Lake Yssel polders almost exclusively occurs as ammonium. Under these conditions ammonium supply by means of mineralization of organic matter takes place very slowly.

In order to obtain information about the nitrogen uptake by *S. congestus*, the ammonium contents of soils covered with dead stems of the first generation and of bare soils were compared, as is shown in Table VIII. The latter remained bare as a result of a permanent cover with seepage water to a depth of at least 2 cm. The sample plots 1, 2 and 3 on bare soils were about 2 m away from the dead vegetations 1, 2 and 3 respectively, while the bare and the originally overgrown plots of each number only differed to a small extent in lutum and

humus percentages (per 100 g dry matter), in g NaCl per l soil moisture and in pH.

On the three *S. congestus* habitats of Table VIII in the uppermost 60 cm, where the roots are found, the mineral nitrogen contents usually proved to be low in comparison with the bare fields. Nevertheless, it is questionable whether the figures of the bare soils might be considered as actual data for the three *S. congestus* habitats at the moment when the first generation started its development. In any case, Table VIII clearly shows that during the growth of these heavy vegetations the mineral nitrogen level of the uppermost 60 cm must have decreased considerably.

Meanwhile, by studying the nitrogen content of the species, it appeared that the uptake of mineral nitrogen by the first generation must take place on a large scale. From Table IV it can be seen that in Eastern Flevoland the aerial parts of the first generation produced meanly 10.000 kg (9.000–11.000 kg) dry organic matter per ha. Its mean nitrogen content amounted to 185 kg per ha. The adventitious roots contained about 2500 kg dry matter per ha, with 35 kg nitrogen. In other words the uptake of the first generation amounted to meanly 220 kg nitrogen per ha.

According to SMITS (1953), in the lutum-range 18–36 % the apparent density (dry volume-weight) of young marine sediments, as has been recorded in Table VIII, lies between 0.86 and 0.58. And so in this case the dry-weight per ha of the uppermost 60 cm varies from 6×10^6 (the soil volume in l per ha) $\times 0.86 = 5.16 \times 10^6$ kg to $6 \times 10^6 \times 0.58 = 3.48 \times 10^6$ kg. In other words, in the lutum-range 18–36 % 1 mg mineral nitrogen per kg dry matter of the uppermost 60 cm corresponds with 5.16 to 3.48 kg mineral nitrogen per ha of the same layer. It follows now that in the lutum-range 18–36 % a vigorous growth of *S. congestus* may be achieved if the species could assimilate $220/5.16 = 42.6$ mg to $220/3.48 = 63.2$ mg mineral nitrogen per kg dry matter from the uppermost 60 cm of the soil. In view of the fact that the soil-layers of the plots from Table VIII are within this lutum-range, it appears that the required mineral nitrogen level did not occur on the plots with the died off vegetations. But on the bare soils it might even be exceeded.

In view of these results the conclusion may be drawn that the rapid vitality decline mainly depends on the strong decrease of the ammonium content in the rhizosphere. Apparently a considerable amount of this nitrogen may be embodied in the first generation of *S. congestus*.

The question arises now what might be the explanation of the high vitality of the second generation growing in some seepage pools during the autumn of 1959. These pools dried out for the first time in June and July and in the uppermost 20–30 cm of the soil aeration had taken place to some extent. But in August the soil surface became wet again (see 4) and so the seed was capable of germination. Although not analysed, it seems possible that mineral nitrogen was liberated from organic matter owing to temporary aeration. It is noteworthy in this connection that under laboratory conditions the soils in Eastern

Flevoland showed a considerable nitrogen mineralization after the first generation had died off.

9. Conclusion

The exceptional opportunity arose in 1959, when it was possible to collect data on the flying-distance of *S. congestus* diaspores. Usually long-distance dissemination will be overlooked owing to the small number of diaspores taking part in this phenomenon. However, on the basis of the observations in 1959 it is likely that anemochorous dissemination over long-distances must be the explanation of the rapid appearance on bare and wet soils, far remote from fruiting sources.

Besides it should be noted that the occurrence of large fruiting *S. congestus* sources in reclaimed areas of the Netherlands might have caused the temporary increase in the adjacent countries in the past, e.g. after the Haarlemmermeer had been drained dry in 1852. Therefore it might be desirable now to look at the species in W. Germany, Belgium, N. France and even in Great Britain.

It is true that the majority of the plants which established themselves in the summer of 1959 nearly everywhere in the Netherlands (Fig. 4), were killed before flowering in 1960, mainly owing to the cleaning of water courses by men and the rising of the water level of the habitats. But on account of the large-scale establishment, it is likely that flowering plants will still be found frequently in the spring of 1960.

It was found that growing as a pioneer on wet and bare soils a single established plant is capable of producing 150.000–200.000 germinative seeds, even in the nearly complete absence of pollinating insects. So a considerable increase might be expected in the next year. That this is true was observed in Eastern Flevoland, where the vast *S. congestus* vegetation arose from seed produced by small sources, which had established themselves in the border area before.

On the basis of this study the conclusion may be drawn that the high vitality of the first generation in the Lake Yssel polders should be attributed to the favourable conditions for germination, seedling growth and further development shortly after the soils had been drained dry. In other words *S. congestus* is a species well 'adapted' to the extreme pioneer conditions prevailing at that time. The ephemeric character is connected with changes in the edaphon under the influence of the vegetation.

SUMMARY

1. In order to find an explanation for the rapid establishment and the ephemeric occurrence of *S. congestus* vegetations in the Lake Yssel polders several ecological characteristics of the winter annual life-form of the species were studied. In this area only the winter annual plays an important part in the vegetation.
2. On five sample plots in Eastern Flevoland the vegetation-cycle of *S. congestus* was studied in the years 1957–1959. In view of these observations we may suppose that the decline in vitality should mainly be attributed to changes in the edaphon under the influence of the first *S. congestus* generation. Apart from *S. congestus* in Eastern Flevoland *Ranunculus sceleratus* showed the highest degree of presence in the vigorous vegetations of the first species.

3. Taking seed output, dry matter production, stem height and number of flower stalks at base of stems as criteria, the vitality of the first generation of *S. congestus* in Eastern Flevoland proved to be high. Usually, the second generation showed a considerable decline in vitality.
4. It was observed that the diaspores may be disseminated over long distances by wind and convection currents, viz. at least 90 km and in all probability as far as 200 km. This must be the explanation of the rapid appearance in new and well suited habitats, far remote from fruiting sources of the species, e.g. in 1959. As to dissemination over short distances, it was found that hydatochorous transport of diaspores may be effective as well.
5. The seed of the winter annual life-form loses viability within three months after dissemination owing to high temperatures and moisture contents. On account of rather low temperatures, seed of the autumn flowering summer annuals may survive the winter.
   Wet soil surfaces appeared to be favourable to germination. Moreover, these soils ought to be muddy and bare for a vigorous growth of seedlings. It seems possible that the NaCl-tolerance of the germination- and seedling-stage depends on the mineral nitrogen content of the soil.
6. Beside the wetness of the soil, the high vitality of the first generation should be attributed to the high mineral nitrogen content of the soil at the time of its emergence from the water. The rapid decline in vitality of the next generation is due to the decrease of the mineral nitrogen content of the soil under the influence of the first generation.

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BOOK REVIEWS

OF PUBLICATIONS RELATED TO BOTANICAL WORK IN THE NETHERLANDS

ZEIJLSTRA Fzn., H. H.: Melchior Treub — Pioneer of a new Era in the History of the Malay Archipelago, 128 p., illus. Amsterdam, 1959, Kon. Instituut voor de Tropen.

Well informed as we may be about the life and work of MELCHIOR TREUB (1851–1910) through a number of obituary notices, biographical sketches, and several, often extensive historical accounts of natural science in the former Dutch East Indies (particularly the botanic gardens, the Department of Agriculture, and the various non-governmental experiment stations), hitherto we lacked a more extensive Treub biography. I think that we must appreciate it that Dr. ZEIJLSTRA did not attempt anything like a definite biography of Treub which would have been a huge undertaking involving an enormous amount of research in Dutch, Indonesian, and other archives, but that he did restrict himself to an extensive biographical memoir. This biography, the value of which is heightened by the fact that the author was associated with Treub and that he received considerable aid in the form of nine ms. essays ("Reminiscences of Melchior Treub") from the late Dr. J. C. KONINGSBERGER (whose son, Prof. V. J. KONINGSBERGER, again was instrumental in arranging for the publication of this memoir), not only gives us a picture of Treub's life and work, but also of the history of the Buitenzorg Botanic Gardens and manifold other activities in the former Dutch East Indies with which TREUB was concerned.

It is a nostalgic picture — TREUB made a world institution of the Buitenzorg Botanic Gardens and its directorate into the most importent and most influential biological position in the Netherlands Empire of his period. He was a hard worker; the personal assistance which he gave to scientific visitors from abroad prior to their trip to Java, while they had their headquarters at Buitenzorg or its Tjibodas annex, and also after they had left (particularly in connection with the publication of the results of their work), was often very extensive.

TREUB's contributions to the science of botany were numerous and are well known. The help given to others and his own scientific interests, however, are all facets of a many-sided policy guided by his basic idea that science (in this case, mainly biology and agriculture), as an anonymous essayist (Gard. Chron. 1910) once expressed it, " . . . was not an end in itself but an instrument to be used . . . to give real effect to that idea, science must lead and not be merely an occasional ally, in the practical affairs of this world. To carry out his dominating idea, he addressed himself with equal energy to two great tasks: one, to make of Buitenzorg the tropical Mecca, whither all the world's botanists should desire to make pilgrimage; the other, to rear a great Agricultural Department for Java, based on scientific knowledge, and conducted by scientific men."

How TREUB accomplished these tasks and became both one of the world's

great biologists and pioneers of the "technical assistance" concept, the author outlines in 6 well balanced chapters:

(1) Early years and university training. Leiden period. 's Lands Plantentuin at Buitenzorg before TREUB's arrival.
(2) TREUB's first year at Buitenzorg. His own investigations. BURCK's arrival.
(3) TREUB as promotor of scientific research in the Netherlands Indies.
(4) TREUB's merits for the development of agriculture in the Netherlands Indies.
(5) TREUB as founder and first Director of the Department of Agriculture in the Netherlands Indies.
(6) TREUB's personality.

The book also includes a genealogy of the TREUB family, a list of visiting scientists who worked at the Hortus Bogoriensis, etc. We miss, however, an index, particularly of personal and place names (available at the Utrecht Biohistorical Institute), a bibliography of TREUB's writings (though most of these will be found in the terminal notes) and a picture of that intriguing liverwort genus *Treubia* (which would have made a nice vignette for the title page). The list of sources might perhaps have been extended to include such biographies as those by BOERLAGE, FAIRCHILD, GOEBEL, GUIGNARD, LOTSY, SCHRÖTER, SOLMS-LAUBACH and TISCHLER which the author will have utilized.

Inasmuch as TREUB was a pioneer of truly international standing, it would be interesting to have an analysis of the influence he exercized on other botanic gardens, biological stations, and the organization of agricultural research, questions admittedly beyond the scope of this stimulating memoir.

FR. V.

FLORA MALESIANA, series II. Pteridophyta. Edited by R. E. HOLTTUM, Kew. Vol. 1, part 1. Dec. 1959, pp. i–xxiv, 1–64, 32 fig. Erven P. Noordhoff, Groningen. Dfl. 10.—, £ 1.–/–.

This represents the start of the revision of the *Pteridophyta* of Malaysia which is edited by Dr. R. E. HOLTTUM, former director of the Botanic Gardens, Singapore, and lately professor at the University of Singapore. HOLTTUM, who has during three decades acquired a unique field knowledge in Malaysia, has agreed to make this his magnum opus which will keep him busy for the next ten years at least, even with the collaboration of some colleagues. The present part is executed in the same way as the spermatophyte series and is entirely his work, with the exception of the *Isoetaceae*, which are by the late Dr. ALSTON. Besides the revisions of the families *Gleicheniaceae* and *Schizaeaceae*, HOLTTUM has found fit to introduce the volume by general chapters, on the morphology of ferns and a bibliography on Malaysian ferns published subsequent to that in the last part of CHRISTENSEN's Index Filicum. Very important are further a modern census of Malaysian genera of *Pteropsida* and two alternative general keys which lead to families or groups of genera. Further there are keys to all genera within their family or group. As there is still no agreement as to the vast and heterogeneous assemblage *Polypodiaceae* sens. lat. HOLTTUM has provisionally accepted two families, *Polypodiaceae* and *Grammatidaceae*, and has divided the rest into groups of genera which seem to him natural taxa, neither specifying their status nor giving them a name. He wishes to postpone his final system till the end of the volume. In the elaboration he keeps in close

touch wich anatomists and cytogeneticists and naturally refers to fossil ferns. When completed this work will represent a major contribution to pteridology.

v. St.

Palynologie — Bibliographie 1959. Service d'information géologique du Bureau de Recherches géologiques et minières. 74, Rue de la Fédération. Paris XVe.

The fourth volume of this annual report contains, this time, only corrections of the list of addresses inserted in the previous volume, changes of addresses, and the names and addresses of palynologists not figuring in the three previous volumes. It opens with informations of bibliographical cards on palynology, delivered monthly to subscribers by the "Service d'Information Géologique", Paris.

F. P. J.

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THE CULTIVATION OF PLANT TISSUES IN VITRO WITH STARCH AS A SOURCE OF CARBON

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Introduction

The literature on plant tissue culture shows few reports on the use of starch as a source of carbon. Gautheret (1945), considering the action of chemical substances on living systems, points out the necessity of putting three questions: *a*, is the substance to be studied indispensable? *b*, what is the optimal concentration? *c*, is substitution by other substances possible? In plant tissue culture, sugars (monosaccharides or disaccharides) proved to be necessary to growth, and after studying the optimal concentration Gautheret turned to the third question and investigated a number of substances for their value as a sugar substitute. These last investigations proved starch to be valueless as a substitute with the tissue tested, i.e. his well-known strain of carrot root tissue.

Hildebrandt and Riker (1946, 1949) investigated a large number of substances for the same purpose. The strains used were derived from tumours of *Tagetes erecta* L. var., *Chrysanthemum frutescens* L. var., *Vinca rosea* L., *Helianthus annuus* L. var. and of a hybrid of *Nicotiana glauca* Grah. ♀ × *N. langsdorffii* Weinm. ♂. According to these authors, little or no growth took place on culture media containing 1 % of soluble starch. Their data indicate, however, that the cultures of *Vinca* and *Nicotiana* tissues grew better than the others. The same results were obtained in experiments on the use of series of concentrations other than 1 %. (Hildebrandt and Riker, 1950, 1953; Riker and Hildebrandt, 1953, 1955). Neither Gautheret nor Riker and Hildebrandt mention attempts to continue their culture on starch media.

The results communicated by Nickell and Burkholder (1950) are entirely different. These authors state that soluble starch is "a surprisingly good source of carbon" for a tissue strain derived from virus tumours of roots of *Rumex acetosa* L. (Burkholder and Nickell, 1949). The growth value, i.e. the ratio between the fresh weights of the cultivated tissues at the beginning and at the end of a certain culture period on a starch medium is about 55 % of that on an optimal glucose medium: 8.56 on glucose against 4.70 on starch medium. Furthermore, prolonged cultivation on starch media by means of subcultures proved to be possible.

The fact that with the iodine reaction a colourless zone around the cultivated tissues becomes apparent led to the presumption that virus tumour tissue of *Rumex acetosa* is able to digest starch by means of

an extra-cellular enzyme. A possibly related phenomenon was discovered by GALL (1948). During an investigation of the influence of 2–4-dichlorophenoxyacetic acid on the transformation of reserve starch in callus-growing fragments of bean stems, he discovered a positive correlation between the intensities of callus formation and of starch hydrolysis. BRAKKE and NICKELL (1951) thoroughly investigated the transformation of starch by *Rumex*-tissue and arrived at two important conclusions. In the first place they were able to show that the starch-splitting enzyme is an α-amylase. Secondly, it seems hardly to be doubted that the enzyme enters the culture medium as a result of a true secretion by intact cells. Some further publications of BRAKKE and NICKELL (1952, 1955) and NICKELL (1953) contain complimentary data. The data given by NICKELL and BRAKKE in 1954 are important for the present investigation. These authors compared the growing properties of their *Rumex*-strain with tissue cultures derived from crown-gall of *Nicotiana tabacum* L., *Helianthus annuus* L. and *Vinca rosea* L. The growth values of *Nicotiana* and *Vinca* tissue were 50.7 and 46.6 %, respectively, of that of *Rumex* tissue, while two sunflower strains exhibited a growth value of only 11 %. This shows that *Nicotiana* and *Vinca* tissues grow tolerably well on starch medium. Therefore, the present writers do not agree with the statement made by Nickell and Brakke that "reports in the literature that other plant tissues studied grow very little or not at all on starch is confirmed here for 4 different crown-gall tissues". Furthermore, no attempts at prolonged culture seem to have been undertaken. LAMPTON (1952) and STRAUS and LA RUE (1954) reported that tissue cultures derived from endosperm of *Asimina triloba* Dunal. and *Zea mays* L. are able to use starch as a source of carbon. After completion of this manuscript, an article by CONSTABEL (1960) was published. This author mentions the fact that tissue cultures derived from *Juniperus communis*, *Crataegus monogyna* and *Pyrus communis* exhibit some growth on starch-containing media. Very good growth was exhibited by a fast growing, auxin autotrophic strain of *Juniperus*. From the description it appears that both α- and β-amylase are secreted although the author does not stress this point, nor is it clear whether prolonged cultivation on starch-containing media was attempted. Finally, attention is drawn to the survey done by GAUTHERET (1955) on the nutrition of plant tissue cultures.

Unaware at the time of the work of Nickell and his co-workers, [1]) the present investigation was started in 1953.

MATERIAL AND METHODS

Seven strains were tested for their ability to use starch as a carbon source. Particulars about the strains used are assembled in Table I. All strains used were well established, and are still in cultivation at our laboratory.

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[1]) The senior author first became acquainted with Nickell's work at the Colloquium on Plant Tissue Culture at Briançon (1954).

TABLE I

Strains derived from:	Isolated by:		No. of passage used:	Particulars:
Cissus spec.	Oort [1])	(1948)	32	cambial zone, twigs
Crataegus monogyna Jacq.	Karstens	(1949)	19	cambial zone, twigs
Daucus carota L.	Gautheret	(1939)	96	cambial zone, root
Daucus carota L.	Karstens	(1952)	18	cambial zone, root
Nicotiana tabacum L.	Morel	(1948)	45	crown-gall
Rubus fruticosus L.	Oort [1])	(1948)	31	cambial zone, twigs
Rubus fruticosus L.	Oort [1]), Karstens	(1951)	32	mutant "accoutumé"

In the culture media used, glucose was replaced by soluble starch. For the first subculture on starch medium a concentration of 5 % soluble starch (Brocades and Stheemann) was used. However, this brand proved to contain an appreciable amount of reducing sugars. It was therefore replaced by soluble starch "Analar" from B.D.H., initially in a concentration of 0.5 % to obtain a clear culture medium. Growth was considerably improved, however, by increasing the concentration to 2 %.

Autoclaving was at first omitted for fear of the production of sugars and other substances by partial hydrolysis of the starch. It was found, however, that under the conditions used no hydrolysis of starch occurs. NICKELL and BURKHOLDER (1950) mention the same experience.

The culture tubes were placed at 25° C in specially constructed incubators. [2]) By means of a small electromotor in these incubators, platforms with culture tubes fixed in a vertical position were slowly rotated. Parallel to the axis of rotation Philips fluorescent tubes were fixed in order to provide a uniform amount of illumination for the cultures.

CULTIVATION ON A STARCH MEDIUM

It very soon became apparent that the seven strains used for this investigation reacted very differently to the transfer from the glucose medium to the starch-containing media. The tissues of *Cissus spec.* and *Crataegus monogyna* exhibit so little growth that a second subculture could not be effected. The five remaining strains showed better growth, so that a second subculture could be made. Gautheret's carrot strain and the *Rubus* "accoutumé" strain showed hardly any growth at all, while our own carrot strain exhibited a slightly better development. The results with the normal strain of *Rubus* were much better, especially with the strain derived from tobacco crown-gall. The experiments were continued with these two tissue cultures. This does not imply that the discarded strains are absolutely unable to develop on starch

[1]) Professor A. J. P. Oort (Wageningen) very kindly presented me with tissue cultures isolated by him during his stay in 1948 in Gautheret's laboratory (Paris).

[2]) We should like to thank Dr. W. Kruyt, Research laboratory of the Amsterdamse Chininefabriek (Amsterdam Quinine Factory) for his kind permission to use the idea of rotating platforms in the construction of our incubators.

media. As a matter of fact, it is our impression that many strains are able to adapt themselves sooner or later to this type of culture medium.

As mentioned before, the crown-gall strain of *Nicotiana* in particular, grows very well on starch. Substrains started in 1953 and cultivated since then on starch, have grown through 42 and 35 passages. Normal tissue of *Rubus fruticosus* can also be grown on starch medium, but the growth is very much slower than is the case with tobacco. Started at the end of 1953, it has grown through only 28 passages. This is partly due to the fact that in the beginning 0.5 % starch was used. In that period growth was so slow that subculturing was only necessary once in four months. Since July 1956, 2 % starch has been used with the result that growth has improved considerably as may be seen from the fact that subcultures now have to be made every three months. In comparison with the tobacco strain, this is still very slow, since the latter has to be transferred to new culture medium every six or eight weeks. This is remarkable because the growth rate of both strains on glucose media is about the same and such that subcultures have to be made every two months. In addition to culture on starch medium in the light, it was investigated whether growth is possible on starch medium in the dark. For this purpose starch substrains of *Nicotiana* were used. Culture proved possible, and two substrains were obtained which grow very well under the conditions described. Subculturing takes place every 2 to $2^1/_2$ months. Started in September 1954, one of these substrains has been cultivated through 31 passages.

Growth on starch medium in comparison with the development on glucose medium

To evaluate the development of tissue cultures on starch medium compared with that on media with glucose, fresh weight and dry weight determinations were carried out on tissue cultures at the beginning and at the end of certain growth periods. To obtain as much uniformity in growth as possible, great care was taken to start with uniform tissue fragments. As material, *Nicotiana* and *Rubus* starch strains were used which had gone through a considerable number of passages. In the case of *Nicotiana*, in addition to experiments in light a few series of dark experiments were done. The results are shown in Table II.

Several points of interest present themselves. In the case of *Nicotiana* it is clear that the growth rate on starch media both in light and darkness is very high, taking into account the fact that the culture period is only 49 days as against 71 days for the cultures on glucose media. Furthermore, it is apparent that growth on starch medium in darkness is very uniform. A second point of interest lies in the fact that the dry-matter content of the tissues grown on starch media is considerably lower than that of tissue cultivated on glucose media. The high water content and the rapid development on starch media are certainly interrelated. A third point to be considered is the striking difference in dry-matter content at the beginning and at the end of

TABLE II

	Number of days cultivation	Growth value [1]		Dry weight as a percentage of fresh weight: at the beginning of the experiment		at the end of the experiment	
Nicotiana tabacum							
crown-gall							
glucose 2 %, light	71	16.4 ± 0.8	n = 20	4.8 (4.4–5.4)	n = 6	6.5 (5.9–8.0)	n = 7
glucose 2 %, dark	71	11.4 ± 1.3	n = 15	6.3 (5.4–7.0)	n = 6	7.2 (5.6–8.0)	n = 7
starch 2 %, light	49	14.2 ± 1.2	n = 24	2.6 (2.1–2.8)	n = 6	2.4 (1.9–3.1)	n = 7
starch 2 %, dark	49	11.0 ± 0.2	n = 24	2.6 (2.1–2.9)	n = 6	2.6 (2.1–3.2)	n = 6
Rubus fruticosus							
glucose 2 %, light	61	13.7 ± 0.6	n = 23	5.5 (5.1–5.8)	n = 6	5.5 (5.2–6.0)	n = 8
starch 2 %, light	61	5.9 ± 0.4	n = 24	3.3 (2.1–3.9)	n = 6	4.3 (3.7–4.9)	n = 8

[1] Ratio of fresh weights at the beginning and at the end of the experiment.

the experiments in tissues cultivated on glucose media. No difference in this respect could be observed in the starch cultures. In the next section this point will be discussed further.

As to the *Rubus* cultures, the low growth value on starch medium is apparent. The difference in dry-matter content between glucose and starch cultures is less spectacular than in *Nicotiana* but is clearly evident.

Growth pattern on glucose and starch media

On transfer of tissue cultures of both *Nicotiana* and *Rubus* from glucose to starch media, striking changes in growth occured. To the best of our knowledge, no mention of this phenomenon has been made in literature. It is, however, so striking that, especially in the case of *Nicotiana*, it is hardly believable that the cultures on glucose and starch belong to the same strain. Crown-gall tissue in vitro from *Nicotiana* appears on glucose medium and cultivated in light as white, semiglobular, downy, solid masses of tissue, growing with a smooth underside on the surface of the culture medium. Grown in darkness, the cultures appear somewhat less regular in shape but otherwise show no differences. Cultivated on starch medium in the presence of light, the colour of the cultures is grass-green and the shape is very irregular. Furthermore, the cultures very often contain cavities, grow to a considerable extent into the culture medium, and are very juicy. Brought back on glucose-containing media, the original growth picture sooner or later returns. Cultivated in darkness, the cultures are pale brownish-yellow in colour and of a very soft substance.

The glucose and starch cultures of *Rubus* show a different picture. On glucose media the cultures are green and show a semiglobular underside that grows well into the medium. Furthermore, the cultures are of a firm consistency. On starch-containing media, however, the cultures are creamy-white in colour, grow on but not into the culture medium, and are of a much softer consistency. More detailed information concerning growth pattern and internal structure will be given in a subsequent paper (Van Lith–Vroom, Gottenbos, and Karstens, 1960).

The cause of the growth observed on starch media

It is obvious that the positive results obtained with substitution of starch for glucose would lead to an investigation of the cause of this phenomenon. It is also obvious that the presence and activity of starch-hydrolyzing enzymes had to be considered.

In our survey of the literature, the work of Brakke and Nickell (1951) is mentioned. Their investigations were able to prove that tissue cultures derived from virus tumours of *Rumex*-roots developed very well with starch as a carbon source as a substitute for glucose because of the activity of an α-amylase actively secreted by the tissue into the culture medium. In the discussion following the papers given by Dr. Nickell at the colloquium at Briançon (Brakke and Nickell, 1955) the senior author stated that in the case of the tissue cultures

of *Nicotiana* and *Rubus* not only α-amylase but probably also β-amylase played a part in the hydrolysis of starch.

Some details of the present research may be given here. Under the influence of the tissues under cultivation the starch present in the culture media disappears in the area close to the tissue. This could be demonstrated by the iodine test for starch. In order to learn more about the agents present, a series of experiments was started using WIJSMAN's diffusion method (1889), applied in more recent time by VAN KLINKENBERG (1931) and MEEUSE (1943, 1952). The principle of Wijsman's test method is very simple. On the surface of a starch solution solidified by means of agar a drop containing starch-hydrolizing enzymes, e.g. malt-extract, is put. In the course of time, the surface is treated with an aqueous iodine solution. According to the presence of one or more types of amylases, a specific colour pattern develops. In the case of α-amylase, for instance, starch is quickly hydrolised into products that do not give any colouration with iodine. The result is a colourless circular area in the middle of a blue field, the colourless area being caused by enzyme activity, the blue surrounding parts showing where starch is still intact. If, however, only β-amylase is present in the test solution, starch hydrolysis is slower and products are formed which give a reddish violet colouration with iodine. In the case of β-amylase, a reddish violet spot surrounded by blue results. In the case of a mixture of α- and β-amylases, a combination of the described results becomes apparent. Around a colourless area caused by the action of α-amylase, first a reddish violet ring (β-amylase activity) and then a blue field (indicating intact starch) become visible.

The material to be tested for amylase activity was used in three ways: as living tissue, as tissue killed by various means, and as press sap. Living tissue was also used in three forms: freshly cut cubes, cubes washed with sterile water to remove the contents of cut cells, and cubes after a period of cultivation. In the last case the cubes were covered with a layer of newly formed cells. By means of small stainless steel hooks implanted into the cubes during the manipulations for making subcultures, it was possible to handle the young cultures without damage. The intact upper side was finally brought into contact with the test plate, with the hope that the cells in question had remained undamaged.

Three methods were used to kill tissue: freezing at a temperature of minus 5° C, treatment with ether vapour, and heating in a water bath at 100° C. Press sap was prepared by forcing a piston into a perforated hollow cylinder containing the material. The sap obtained was used without further treatment or treated with different temperatures. For the tissue-test, *Nicotiana* tissue was used, while press sap was prepared from *Nicotiana*, *Rubus* normal and *Daucus carota* Gautheret's strain, all tissues being cultivated on glucose medium. Amylase appeared to be active in the case of living tissue and of untreated press sap.

In order to obtain more information about the amylases present,

TABLE III

Material used	Unheated control	Heated to 70° C	Heated to 80° C	Heated to 100° C
Nicotiana tabacum crown-gall	large colourless centre reddish violet ring	smaller colourless centre broader reddish ring	pale violet centre reddish violet ring	no decolouration
Daucus carota strain Gautheret	colourless centre reddish violet ring	yellow brown centre reddish violet ring	diffuse, pale violet spot	no decolouration
Rubus fruticosus normal	brownish violet centre reddish violet ring	diffuse, pale violet spot	no decolouration	no decolouration

a number of experiments were done on temperature sensitivity. In addition to untreated press sap, sap was tested after treatment in a waterbath for 10 minutes at 70, 80, and 100° C. The results are collected in Table III.

It is apparent that quantitative and qualitative differences in the constitution of the press sap samples affect each other. Furthermore, it might be possible that certain press saps contain interfering impurities. From our data it seems that the activity of α-amylase decreases considerably with the 70° C treatment while that of β-amylase remained the same. This is in contradiction to the results of VAN KLINKENBERG (1931) who using purified preparations came to the conclusion that β-amylase is the less stable enzyme. This author investigated the pH influence on the activity of amylases both in a mixture of α-amylase and β-amylase and separately. α-Amylase appears to be active in a pH range of 4–8, while β-amylase shows a much wider range of activity i.e. from 3–11.5.

A plate method was used to test the pH sensitivity of amylase present in tissue cultures. In Petri dishes starch-containing agar plates of the same thickness were prepared. Dilute hydrochloric acid or sodium hydroxyde were used to obtain a series of plates to cover a wide pH range. The pH was measured with the aid of a glass electrode. Two plates were available for each point.

From well-grown tissue cultures of *Nicotiana*, uniform slices were cut by means of a special apparatus; from these slices cylinders were punched out and placed on the test plates. At the end of 48 hours at 25° C the plates were developed with the aid of an iodine solution. The results are summarized in Table IV.

TABLE IV

pH	Colourless central area, diam. in mm.	Pale violet coloured central area, diam. in mm.	Reddish violet ring, thickness in mm.
2.18	—	—	—
2.53	—	—	—
3.03	—	5	—
3.70	—	13	2
4.12	—	17	5
5.13	31	—	4.2
5.33	32	—	3.5
6.27	36	—	4
6.88	35.5	—	4
7.89	36	—	5.5
8.25	—	21	7
8.67	—	30	11.5
9.66	—	21	7
10.23	—	10	2
10.37	—	21	7
10.94	—	14.5	4
11.48	—	9	1

The authors are very well aware that these data do not give much information as to the specific pH ranges of α-amylase and β-amylase

activity in the material tested. The data in columns 2 and 3 possibly point to the combined activity of both α- and β-amylase since the values for the colourless area start and stop very abruptly. It seems probable, however, that the data in column 4 must be ascribed to the presence of β-amylase.

It is necessary to draw attention once more to the problem of whether the presence of one or more amylases in the culture medium is due to a true secretion of these enzymes by intact cells of the cultivated tissue or whether these substances have penetrated accidentally from cells damaged by growth or other processes. The fact that much larger quantities of amylases were found in the culture medium than were present in the tissue itself was a strong argument for Brakke and Nickell (1951) to draw the conclusion, that the observed situation could only be explained as a veritable secretion of the enzymes by intact cells. This point was discussed after the paper read by Nickell at the Briançon colloquium in 1954 (Brakke and Nickell, 1955). On that occasion Gautheret gave it as his opinion that as a result of irregular growth certain cells are pressed and become damaged. From such cells enzymes will penetrate into the culture medium. In his survey on the physiology of plant tissue cultures (Gautheret, 1955), however, the point is no longer stressed. It is our opinion that it will be very difficult to exclude the possibility of accidental penetration of enzymes from damaged cells. The results reported by Brakke and Nickell, however, are at least an indication that real secretion can be very important. The problem of true secretion of enzymes by tissue cultures is also discussed by Reinert, Schraudolf and Tazawa (1957).

Another question is the "reasonableness" of the presence of amylase in culture media. This cannot be discussed before the presence of notable quantities of amylases in culture media without starch is established. The experiments summarized in Table III make such presence very probable. The Wijsman plate method was used to settle this point. Cylinders of starch or glucose-containing culture media on which tissues had been cultivated were put onto starch-containing agar plates. At the end of equal periods of incubation, identical and positive results as to the presence of amylases were obtained. This result makes such "reasonableness" very doubtful. This phenomenon was described long ago in the microbiological literature, among others for *Aspergillus niger* v. Tiegh. (Funke, 1922).

SUMMARY

Experiments on the possibility of cultivating tissue cultures on media containing starch instead of glucose have led to starch strains derived from a crown-gall strain of *Nicotiana tabacum* and from a normal cambium strain of *Rubus fruticosus*. Started in 1953, to date 42 and 28 passages respectively could be accomplished by uninterrupted cultivation on starch media. A substrain of *Nicotiana* on starch medium has been cultivated in darkness through 31 passages. Cultivation on starch media could be accomplished because of the activity of amylases given off by the cultivated tissues. The presence of an α-amylase and possibly a β-amylase could be established. The phenomenon has been described that growth habits on glucose

and starch media are strikingly different. Transfer back to glucose medium results sooner or later in the restoration of the glucose growth habit. In a separate paper, the changes in growth habit, the observed changes in growth pattern, and particulars about the internal structure will be discussed. Biochemical aspects of the phenomenon described are under investigation.

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GENERAL APPEARANCE, GROWTH PATTERN, AND ANATOMICAL STRUCTURE OF CROWN-GALL TISSUE OF NICOTIANA TABACUM L. GROWN IN VITRO ON CULTURE MEDIA CONTAINING GLUCOSE OR SOLUBLE STARCH AS A CARBON SOURCE

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INTRODUCTION

A recent publication (KARSTENS and DE MEESTER–MANGER CATS, 1960) described the observation that substitution of glucose as a carbon source for soluble starch led to a striking alteration in the general appearance of the tissue cultures involved. This phenomenon was observed in a crown-gall strain of *Nicotiana tabacum* L. and in a strain derived from "cambium" (BAILEY, 1943) of *Rubus fruticosus* L. As to the origin of the strains used, that of tobacco was originally isolated by MOREL (1948), while the *Rubus*-strain was received in 1949 by the courtesy of Professor A. J. P. Oort who in 1948 isolated it in Gautheret's laboratory.

The present publication gives details concerning the external shape, growth pattern, and internal structure of tissue cultures of tobacco crown-gall. In addition to observations on tissues grown on glucose and starch media under the influence of light, the same experiments were done in total darkness. Particulars about conditions of culture can be found in the publication by KARSTENS and DE MEESTER–MANGER CATS (1960). In addition, some details will be given concerning the occurence of certain growth types in strains of *Cissus spec.*, *Crataegus monogyna* Jacq., *Daucus carota* L., *Rubus fruticosus* L., (KARSTENS and DE MEESTER–MANGER CATS (1960)) and of *Helianthus tuberosus* L. The strain of *Helianthus tuberosus* was derived from tubers of the cultivar White Jerusalem Artichoke. Details about the origin of the other strains can be found in the publication by Karstens and De Meester–Manger Cats. These are all normal strains and, therefore, to be cultivated on auxin-containing media.

It is remarkable that there is so little in the literature about how the general shape of tissue cultures comes into being, the more so because of the great and interesting variety in shape and growth habit. GAUTHERET (1959, p. 313) describes callus grown from the original explants as generally dense and consisting of firm tissue. Subcultured, spherical, hemi-spherical, or somewhat flattened colonies develop. This fleshy type may be maintained in the course of succes-

sive subcultures but quite often sooner or later other types develop. Gautheret distinguishes lamellar, compound, and discontinuous types of tissue cultures. In his conclusions on this subject (p. 344) the author states that "Les caractères des colonies tissulaires sont à la fois si nets et si variés qu'on pourrait être tenté de les utiliser pour établir une véritable classification des cultures *in vitro*." Only in a few cases has the development in shape of tissue cultures from the cubical fragments cut from the mass of tissue of the former "generation" been followed step by step (Caplin, 1947). More often, casual remarks base on "anatomical snapshots" are encountered.

General appearance and shape of tissue cultures

In order to obtain callus formation in vitro from explants and subculture the newly-formed tissues to establish tissue cultures, media containing glucose or saccharose are nearly always used. This is done because of the simple fact that growth on glucose media, and less often, on media with saccharose, proved to be satisfactory. It is comprehensible, but in fact not reasonable, that there is a tendency to call "normal" all those phenomena which can be observed in tissue cultures grown on one or the other of these two media. The present writers will try to avoid the use of this term.

As has been mentioned in the introduction, crown-gall tissue of *Nicotiana* was cultivated under four different sets of conditions relating to the carbon source used and in relation to cultivation in darkness or in the light. Otherwise the conditions were identical. Plate 1*a* shows the difference in general appearance of glucose and starch cultures while details are presented in Table I.

It seems hardly necessary to comment on the details given in Table I. It is evident that the cultures on glucose media in both light and dark differ in nearly all respects from the starch types. The most important difference between the two starch types lies in the presence of considerable quantities of chlorophyll in the starch-light cultures.

The differences in general appearance, shape of the cultures, etc. were of considerable interest. Since microscopical examination of directional sections cut from tissue cultures is insufficient for an understanding of the development of such cultures, the authors have tried to obtain more information by another method. For this purpose, cubical fragments cut from cultures in the same way as is done for making subcultures were dusted on all sides with finely powdered sterilized charcoal before being put on fresh culture medium. The way in which the fragments develop into their specific shape can be followed to a certain extent with the aid of the charcoal particles. For instance, in certain strains, as growth proceeds the particles become dispersed specifically, more or less evenly, over the expanding surface of the culture. From sections made either by hand or by means of the usual microtechnical methods, the distribution of the charcoal particles can be easily studied. Using this method, several characteristic growth types could be established, some of which are represented in Fig. 1.

TABLE 1

CULTURE MEDIUM	LIGHT OR DARKNESS	SHAPE of the CULTURE	GROWTH in relation to SUBSTRATE	COLOUR	CONSISTENCY
glucose 2 %	light	hemispherical	underside flat, culture on top of substrate	nearly white, opaque, downy	firm, massive
glucose 2 %	darkness	somewhat flattened	underside flat, culture on top of substrate	dirty white, opaque, somewhat fluffy	firm, massive
soluble starch 2 %	light	irregularly lobed	underside irregular, penetrating into substrate	variously shaded grass-green, somewhat translucent, fluffy	juicy, somewhat friable; cavities may be present
soluble starch 2 %	darkness	irregularly lobed	underside irregular, penetrating into substrate	pale brownish yellow, somewhat translucent, fluffy	very juicy and soft; cavities

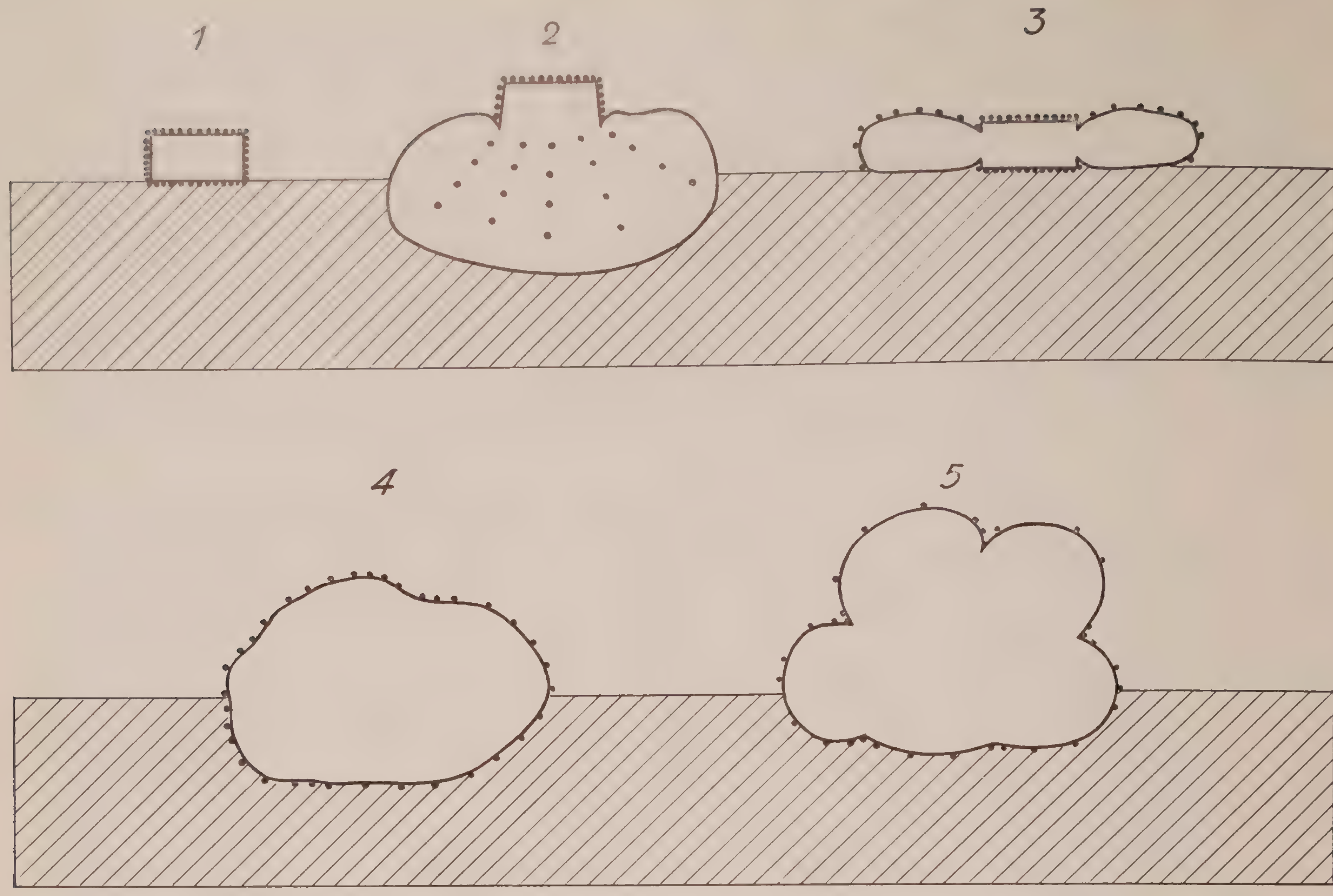

Fig. 1. Schematical representation of growth types. (median sections). The black dots represent charcoal particles with which the initial fragment of the culture was dusted. Culture medium hatched. 1, initial fragment; 2, *Rubus fruticosus*; 3, *Cissus spec.*; 4, *Helianthus tuberosus*; 5, *Daucus carota.*

Certain strains such as those derived from *Rubus fruticosus* L., *Crataegus monogyna* Jacq., and *Cissus spec.* always exhibit the initial fragment of the culture in some way or another. From Fig. 1, Nos. 1, 2, and 3, it is evident that growth can progress along very different lines. In *Rubus*, (Fig. 1, 2.) for instance, the principal growth takes place at the lower surface, while in *Cissus* (Fig. 1, 3.) development is always found along the sides. As a result, the cultures of *Cissus* grow on top of the culture medium, those of *Rubus* partialy penetrate into the substrate. A third difference lies in the fact that in the case of *Cissus* the original fragment always becomes necrotic, while that of *Rubus* remains alive and capable of growth. A fourth difference between the two strains consists of the distribution of the charcoal particles. In the case of *Cissus*, the particles are found at the outside of the growing and non-growing parts of the culture, whereas in *Rubus* the charcoal particles are found not only on the outside of the non-growing part of the culture but also along radiating lines in the interior of the growing part of the culture, starting at the site of the former lower surface of the original fragment. The observations made on *Cissus*-cultures need not to be further discussed since nothing unusual was seen. The case of *Rubus*, however, is much more interesting. The observed distribution of the charcoal particles must develop in such a way that some of the particles are grown over very soon while the rest are pushed forward by growing tissue and are only gradually grown over. This is of interest because it means that the

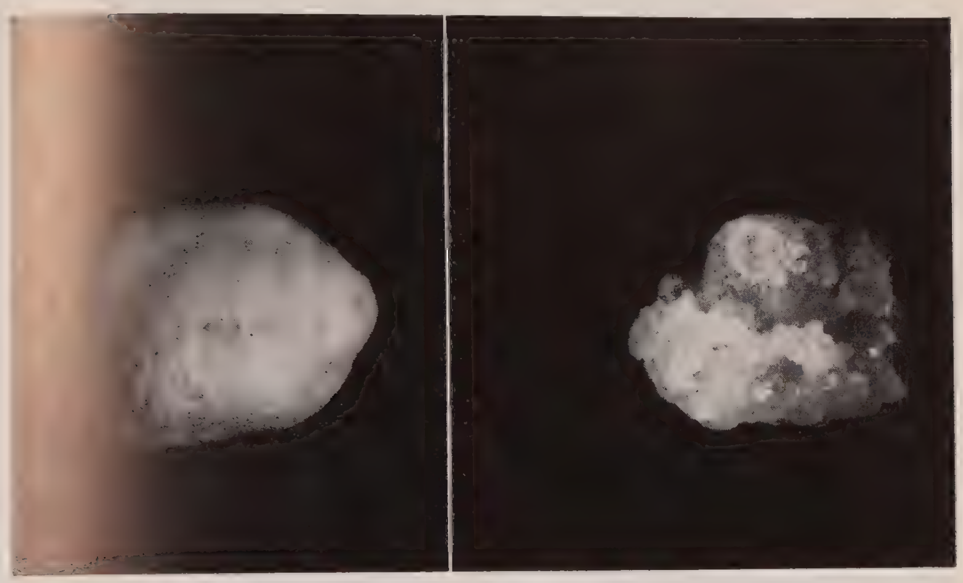

Plate 1*a*. *Nicotiana tabacum*. Crown-gall. Tissue cultures seen from above. To the left, a typical cushion-shaped tissue culture grown on glucose medium; to the right, a culture on starch medium exhibiting the characteristic knotty, somewhat translucent, appearance. (1.5 ×).

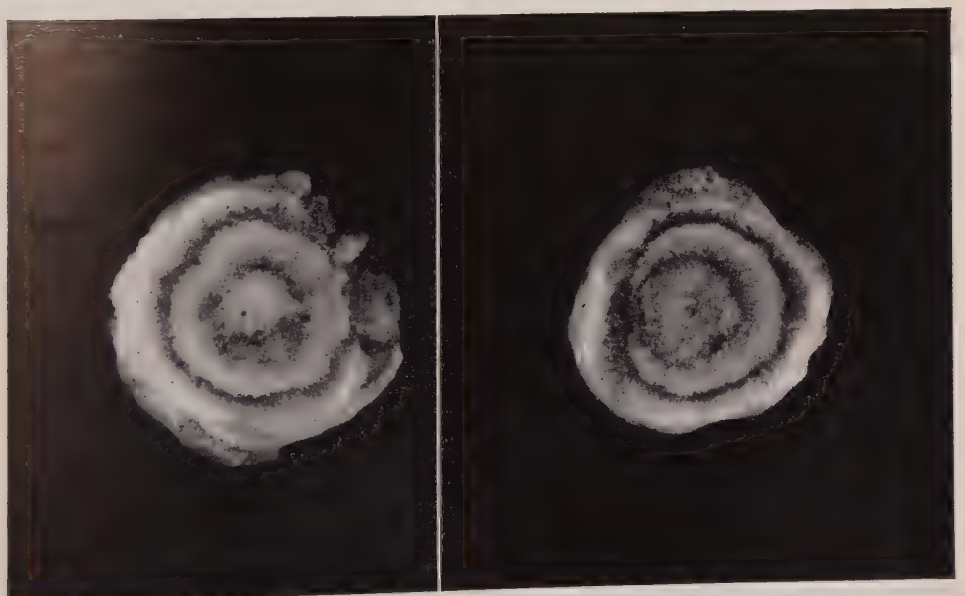

Plate 1*b*. *Nicotiana tabacum*. Crown-gall. Transversely cut cultures derived from cylindrical samples dusted with charcoal. After 26 days of cultivation a second dusting was applied. The photographs were taken 40 days after the second charcoal treatment. Two black zones can be distinguished. In the centre of each culture the little hole left by the stainless steel hook used to handle the material can be seen as a black point. (1.5 ×).

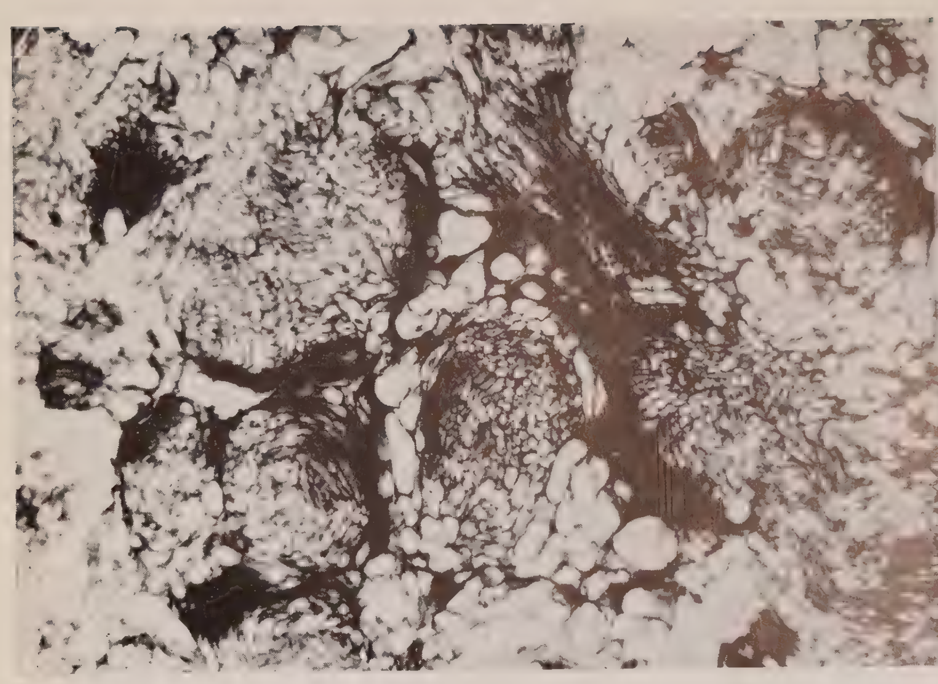

Plate 2*a*. *Nicotiana tabacum*. Crown-gall. Longitudinal section through a culture grown for about eight weeks on glucose medium in the light. The picture shows part of the initial fragment with vascular nodules and a strand of vascular tissue amidst parenchyma. The dark areas represent crushed cell masses. (30 ×)

Plate 2*b*. *Nicotiana tabacum*. Crown-gall. Part of a vascular strand like that of Plate 2*a*, showing lignified reticular elements. (500 ×).

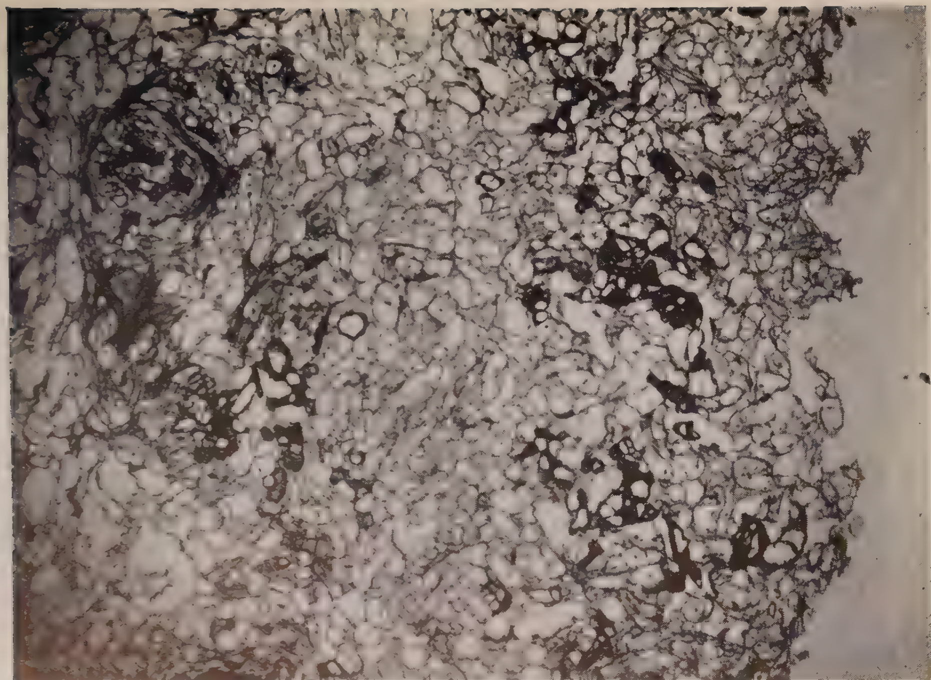

Plate 3*a*. *Nicotiana tabacum*. Crown-gall. Longitudinal section through a culture grown on glucose medium for about eight weeks in the light. To the left a part of the initial fragment with vascular nodules can be seen. All the rest in newly-formed tissue with scattered lignified elements. To the right the margin of the culture is visible. (30 ×).

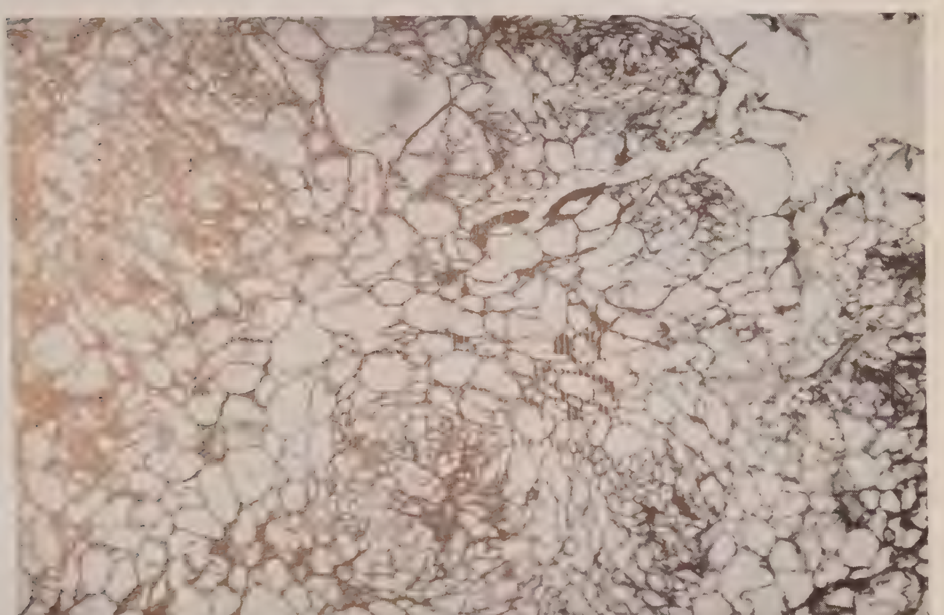

Plate 3*b*. *Nicotiana tabacum*. Crown-gall. Longitudina lsection through a culture grown for about six weeks on starch medium in the light. Among the large-celled parenchyma, scattered nodules formed by meristematic zones are present. Only a few lignified elements can be observed. (43 ×).

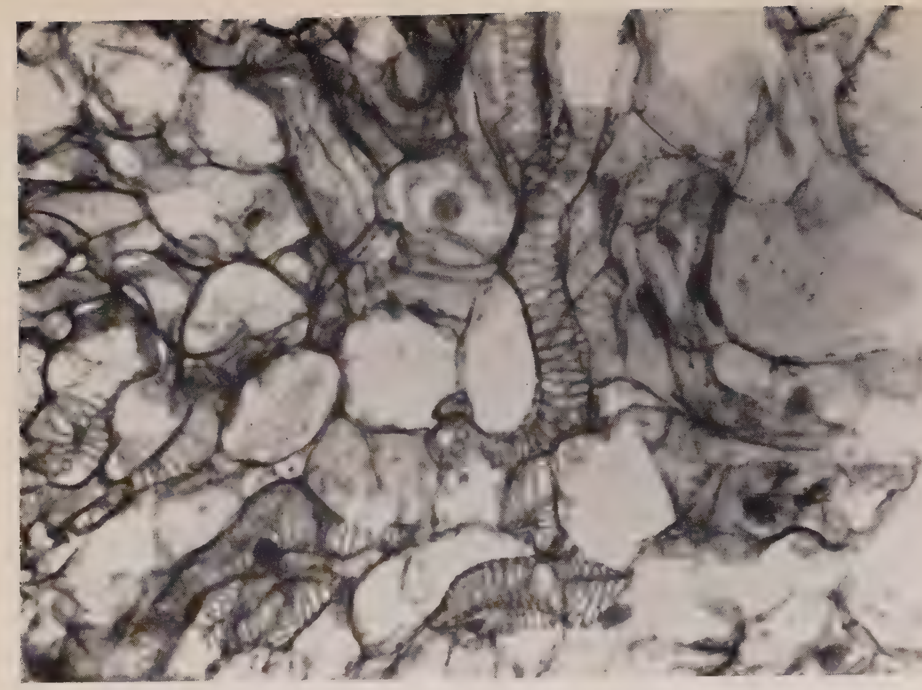

Plate 4*a*. *Nicotiana tabacum*. Crown-gall. Longitudinal section through a culture grown for about six weeks on starch medium in the light. Lignified reticular sometimes more or less scalariform elements. (300 ×).

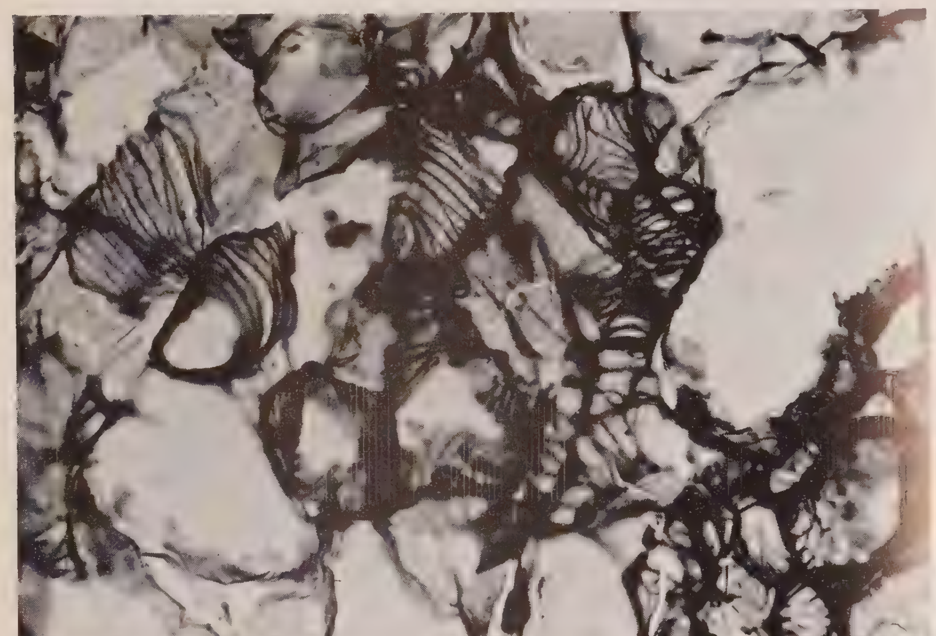

Plate 4*b*. *Nicotiana tabacum*. Crown-gall. Longitudinal section through a cultu grown for about six weeks on starch medium in the dark. The picture shows so lignified elements with the delicate, spiral bands characteristic for this tissue cult grown under the conditions described. (300 ×).

cells of the growing part of the culture do not form a closed front but are able to grow round obstacles.

Totally different growth types are exhibited by the strains of *Helianthus tuberosus* L. and *Daucus carota* L. Both were derived from the tuberous parts of the plants. It should be mentioned here that at the time of the experiments the strains were still of the fleshy type. Later, especially in the case of *Helianthus*, the strains changed more and more into the compound type. Such transformations have been observed before (see GAUTHERET, 1959, p. 319.) The growth types of these two strains are characterized by the pecularity that the original tissue fragment cannot be found in the tissue culture derived from it. (Fig. 1, nos 4 and 5). From the charcoal experiments it appeared that the charcoal particles become more or less uniformly dispersed on the expanding surface of the tissue culture. It is self-evident that the dispersion is greatest on the surface of the fastest growing parts of the cultures. The distribution of the charcoal particles all over the surface of the cultures is caused by the fact that the particles do not become enclosed: in this type of culture, therefore, the peripheral cells form a closed front. Apart from these similarities the strains differ in that the carrot cultures become more or less lobed, while the *Heliantus*-cultures expand more evenly.

The same charcoal method was used to study the growth pattern of the tissue cultures of tobacco crown-gall cultivated under the four

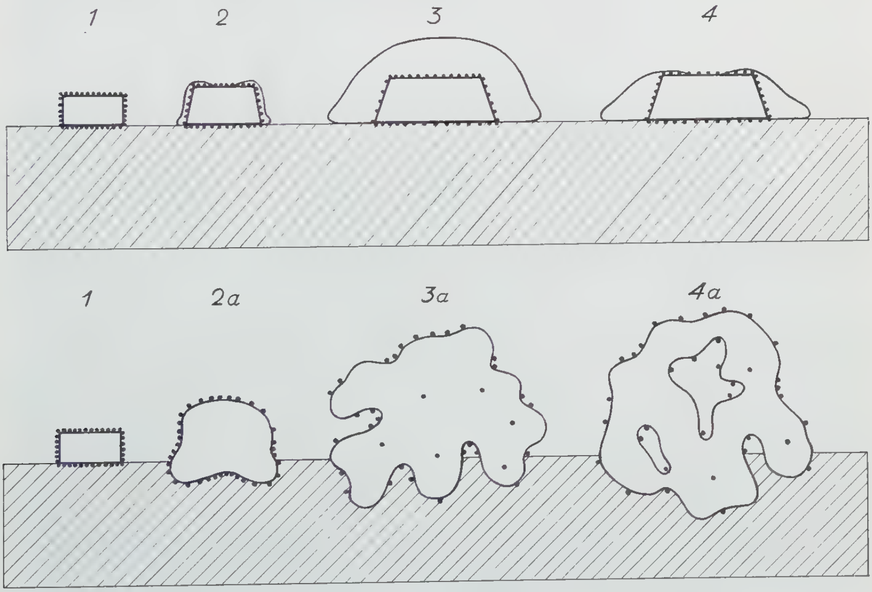

Fig. 2. Schematical representation of the differences in growth type of tobacco crown-gall cultivated *in vitro* (median sections). The black dots represent charcoal particles. Culture medium hatched. 1, initial fragment; 2, and 2*a*, young cultures on glucose and on starch media; 3, glucose-light culture; 4, glucose-dark culture; 3*a*, starch-light culture; 4*a*, starch-dark culture.

sets of conditions described above. The results are given somewhat schematically in Fig. 2.

The first conclusion to be drawn is at the same time a confirmation of the impression obtained by studying a vertical section from a culture grown on a glucose medium in the presence of light: such a section consists of a trapezium shaped area with a paler-coloured border along three sides, creating the impression that the darker area represents the original fragment while the border consists of newly formed tissue. The charcoal experiments leave no doubt that this is indeed the case. The layer of charcoal particles enveloping the initial fragment, with the exception of the lower surface, is broken on all sides by newly-formed peripheral cells growing around the charcoal particles which become, finally, wholy grown over. It must be emphasized that no charcoal particles are carried away, as was observed in the experiments with *Rubus* cultures; they remain together in the form of a flat black layer.

Another point of interest is the observation that the volume of the initial fragment as indicated by the coat of charcoal increases in size (Fig. 2, nos 2 and 3). In the section on internal structure more will be said about this point. During the development described, the charcoal particles disappear more and more under a layer of newly-formed tissue with the result that blackened initial fragments turn into white tissue cultures (Fig. 2, nos 1–3). These in turn can be dusted again with charcoal whereupon the process repeats itself. (Plate 1*b*). It is evident that tissue cultures of tobacco crown-gall grown on glucose media in the presence of light represent a third growth type in which the initial fragment disappears under an overgrowth of new tissue but remains intact in the interior of the colony.

The growth pattern of the glucose-dark cultures may be considered to be a variation of this type. In such cultures only a small quantitiy of new tissue or even none at all is formed at the top of the initial fragment. As a result, the charcoal particles remain visible to a greater or lesser degree at the top of the culture (Fig. 2, no. 4).

A third point to be stressed is the difference in growth pattern between cultures grown on glucose and those from starch media. In sections from glucose cultures the initial fragment is easily detectable, but this is not the case in starch cultures. Furthermore, it became evident that in sharp contrast to the very regular shape of glucose cultures, that of cultures growing on starch media is very irregular. The haphazard distribution of more or less active growth zones in the initial fragment results in a very irregularly lobed culture type. Irregular growth also causes cavities to be formed (Fig. 2, no. 4*a*). As a matter of fact, these cavities, often entirely surrounded by a layer of tissue, are formed by secondary fusion of irregularly expanding lumps of tissue. It is our impression that formation of cavities in cultures on starch media cultivated in light becomes increasingly evident. In early subcultures this tendency was much less in evidence. In dark cultures on starch medium, cavities were observed from the beginning. This irregular growth can be followed fairly well by

studying the distribution of charcoal particles. The haphazard distribution on the outside of the culture, in the interior of dense parts of the cultures, and enclosed in cavities, is only possible with a very irregular type of growth pattern.

Anatomical data

It should be kept in mind in studying the internal structure of tissue cultures, that somewhere among areas of newly-formed tissue these cultures contain, either in concentrated or in dispersed form, remnants of the initial fragment. Furthermore, the anatomical structure of the initial fragment varies with the age of the subculture from which it originates and may vary according to its site in the mother culture. These considerations are based on the observation that the anatomical structure of a tissue culture undergoes a series of changes during each period of growth, from initial fragment to tissue culture ripe for the next subculture.

a. *The anatomical structure of glucose-cultures*

Attention must be drawn anew to the fact that in fresh and unstained sections of cultures grown on media with glucose as a carbon source, in both light and dark, the initial fragment and the newly-formed tissue are macroscopically easily distinguishable. Apart from a difference in colour, the consistency of the two parts is very different. The newly-formed tissue is firm but very easily cut, while the former original fragment is much tougher. The cause of this difference can be demonstrated by treating a section from a one to two months old culture with phloroglucinol and concentrated hydro-chloric acid. The original fragment proves to be composed of a large number of heavily lignified nodules separated by narrow layers of unlignified tissue, while the newly-formed tissue exhibits very little lignification. Microscopically observation reveals the anatomical structure of these nodules. Formed by more or less cup-shaped cambial zones, nodular complexes constituted of neatly arranged tracheary and probably also phloem-like elements, prove to be present. The latter elements are situated at the convex side of the cambial zones. By the continued production of tracheary elements, many of the phloem-like cells, together with adjacent parenchyma-cells from the ground tissue, become compressed into cup-shaped masses of cell-wall material. Occasionally, strand-like formations connecting several of the nodules can be observed (Plate 2*a*). Part of such a strand, exhibiting the reticular lignification characteristic of all tracheary elements present in tobacco crown-gall cultures grown on glucose media is given in Plate 2*b*. The newly-formed tissue consists mainly of parenchymatous cells with few scattered lignified reticular elements, sometimes gathered in small groups. (Plate 3*a*). There appear to be present concentric zones with more or less lignified elements.

b. *The anatomy of cultures grown on starch media*

In a preceding section it was mentioned that in tobacco crown-

gall cultures grown on media with soluble starch as a carbon source, in the light as well as in darkness, the initial fragment becomes dispersed all through the culture. Therefore, the initial tissue and newly-formed tissue cannot be distinguished. It is possible, however, that those parts which contain lignified elements in larger groups are parts of the initial fragments. Tobacco crown-gall tissue cultures grown on starch media under the influence of light or without any light at all have, apart from a few points of difference, the same anatomical constitution. In both cases a loosely built and very disorderly structure can be observed, consisting for the most part of parenchyma. Areas consisting of smaller cells are found amidst others characterized by the presence of much larger cells. In both light and dark cultures, cells of a diameter of 200μ can be often found. Cells of such a diameter are only rarely found in cultures from glucose media. As a matter of fact, the average diameter of cells from cultures of glucose media is much smaller. The small-celled areas in starch cultures can be considered to be of a meristematic character. Scattered throughout the tissue culture, lignified tracheid-like elements, solitary

Fig. 3. Median section of a starch-dark culture of tobacco crown-gall. Lacunar growth type. Culture medium hatched. Each black dot indicates the location of a lignified element.

or in small groups, are present (Fig. 3). The observed structure is very much like that described by White (1939) for his famous tissue culture derived from so-called genetical tumours of the hybrid between *Nicotiana glauca* ♀ and *N. langsdorffi* ♂. Here too, areas consisting of small cells alternate with large-celled zones. "It is not possible", according to White "to identify phloem, cambium, phelloderm, sclerenchyma, nor any other normal cell type except parenchyma, meristem, and an occasional isolated scalariform cell". Still, there

are some points of difference with our starch cultures. Starch cultures grown in the presence of light exhibit somewhat more differentiation because of the rare presence of formations comparable with the heavily lignified nodules in the initial fragment of glucose cultures (Plate 3*b*). However, lignification in this case is restricted to some few elements in the centre. In starch-dark cultures such formations have never been encountered.

White described the presence of scattered scalariform elements. In our starch cultures grown in the presence of light the lignification pattern is nearly always reticular. In addition to more or less rounded cells, much elongated tracheid-like elements are present (Plate 4*a*). Apart from the fact that such elongated elements are extremely rare in starch cultures grown in darkness, the lignification pattern is also quite different. In starch-dark cultures lignification takes place to a lesser degree and is nearly always present in the form of fine spiral bands (Plate 4*b*). Reticular elements are only rarely encountered. As far as the present authors are aware, the type of lignification described here is very seldom found in the literature concerning plant tissue culture. Muir, Hildebrandt and Riker (1958) present some illustrations of tracheid-like cells from a marigold tissue culture (*Tagetes erecta* L, var. "Sunset Giant") which clearly exhibit the same lignification pattern. From the description and illustrations, the marigold tissue culture seems to exhibit many points of resemblance with our tobacco culture cultivated on starch media in the dark. As a matter of fact, both tissue cultures are derived from crown-gall, both are cultivated in the dark, and both appear to have the same juicy consistency.

Discussion

The anatomical study of crown-gall tissue cultures of tobacco grown on media with glucose or starch as a carbon source provides information that agrees very well with previously published results (Karstens and De Meester–Manger Cats, 1960). In that publication, attention was drawn to the fact that dry weight as a percentage of fresh weight in tobacco grown-gall tissue and in cambial tissue of *Rubus fruticosus* L. cultivated in vitro shows a much higher value on glucose media than on media with soluble starch as a carbon source. The data pertinent to the present publication are presented in Table II.

TABLE II

GROWTH CONDITIONS	DAYS OF CULTIVATION	DRY WEIGHT AS A PERCENTAGE OF FRESH WEIGHT	
		at the beginning of the experiment	at the end of the experiment
glucose 2 %, light	71	4.8 (4.4–5.4)	6.5 (5.9–8.0)
glucose 2 %, dark	71	6.3 (5.4–7.0)	7.2 (5.6–8.0)
soluble starch 2 %, light	49	2.6 (2.1–2.8)	2.4 (1.9–3.1)
soluble starch 2 %, dark	49	2.6 (2.1–2.9)	2.6 (2.1–3.2)

It is evident that the very low dry weight value on starch medium is caused by two factors. In the first place, tissues grown on starch media are composed of larger cells with a relatively higher water content. Secondly, the number of cells with thin cell-walls is much larger because the amount of lignification on these media is almost negligible.

Another interesting point is the difference in the percentage of dry weight at the beginning and at the end of the experiment for cultivation on glucose media. On starch media, in strong contrast, the percentages of dry weight of the tissues at the beginning and at the end of the culture period are identical.

These results fit in with the anatomical features observed. In the case of the starch cultures the initial fragment "dissolves" into the new tissue culture. Each fragment cut from such a culture has the same dry weight/fresh weight ratio, because the anatomical structure is statistically uniform throughout. Thus, the initial fragment has the same ratio as the whole culture from which the fragment is derived. Since the pattern of development is maintained during each subculture, the culture present at the end of a new culture period will possess the same properties as its predecessor.

The situation with the cultures on glucose media is totally different. Here, the initial fragment does not "dissolve" into the new culture. On the contrary, it follows a differentiation pattern of its own and exhibits considerable change through the formation of large amounts of lignified cells. The new culture, therefore, does not possess a uniform internal structure, but is composed of two areas of different character, i.e. the former initial fragment containing many lignified elements and a peripheral zone of newly-formed tissue with fewer, less lignified elements. Since the initial fragment is preferably cut from newly-formed tissue, it is obvious that the formation of large amounts of heavily lignified cells in this part of the new culture results in a higher dry weight/fresh weight ratio. The differences between glucose-light and glucose-dark cultures very probably depend on the fact that in the latter case, the zone of newly-formed tissue is thinner, so much thinner that it is not really possible to make subcultures from fragments consisting exclusively of newly-formed tissue. As a result, part of the initial fragment of the former generation will be present in the initial fragment of the new culture. This probably accounts for the higher initial dry weight/fresh weight ratio of the glucose-dark cultures. Finally, the changes which take place in the initial fragment, combined with the relatively poor development of new tissue, account for the higher ratio in "full-grown" glucose-dark cultures over glucose-light ones.

SUMMARY

Substitution of glucose as a carbon source for soluble starch can lead to a striking alteration in the general appearance of tissue cultures. This phenomenon was observed in tobacco crown-gall tissue and in a strain derived from the cambial zone of twigs of *Rubus fruticosus* L. (Karstens and De Meester–Manger Cats, 1960).

In the present paper details are given on the general appearance, growth pattern, and anatomical structure of tobacco crown-gall tissue cultures. In Table I differences of shape, colour, consistency, and manner of growth in relation to external conditions have been summarized. A method is described by which it is possible to study the growth pattern of tissue cultures: initial fragments or young tissue cultures are dusted with powdered charcoal under sterile conditions. After growth has taken place, the localization of the charcoal particles can be studied macroscopically, or microscopically in sections prepared according to routine microtechnical methods. Besides the tobacco cultures on glucose and starch media under varied external conditions, a number of other tissue cultures were tested. As a result, several growth types could be distinguished.

It is a matter of some interest that the tissue cultures of tobacco crown-gall on glucose and starch media possess totally different growth habits. In the glucose cultures the initial fragment remains intact and exhibits a very special differentiation while in the starch cultures the initial fragment "dissolves" into the growing culture. In glucose cultures new growth is strictly localized, i.e. in a meristematic zone formed at the outer surface of the initial fragment, the side in contact with the culture medium being excepted. In starch cultures, however, growth takes place by the activity of scattered meristematic loci present in the initial fragment and afterwards in the whole tissue culture. The initial fragments in glucose cultures, as mentioned before, exhibit a very special differentiation. In the initial fragment a great number of cup-shaped meristematic zones are initiated and give rise to complexes of heavily lignified cells. In the newly-formed tissue lignification takes place to a much lesser extent and in such a way that lignified elements are sparsely present in otherwise parenchymatic tissue.

In starch cultures cup-shaped meristems occur very rarely. They can only be found in light cultures. In addition, few lignified elements are formed by those meristems. On the whole, starch cultures grown either in the light or in the dark have a parenchymatic character with few and very scattered lignified elements. Finally, the lignification pattern of the lignified elements proved to be different in glucose and starch cultures. The anatomical features agree very well with the figures on dry weight/fresh weight ratios given in a previous paper (Karstens and De Meester–Manger Cats, 1960) and summarized in Table II.

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OBSERVATIONS ON CHROMOSOMES IN THE GENUS INDIGOFERA L.

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(*received March 6th, 1960*)

This study on *Indigofera* species mainly from the West African region, will be the first of some publications dealing with the cytology of West African Leguminous plants (except trees) present in the savannah regions. These are plants which might either come into consideration as auxiliary crops or are near relatives of auxiliary plants already in use. Seeds and herbarium material were collected by the author during a two months' tour early in 1957. The tour started at Kano and proceeded via Jos, Samaru, Zinder, Fort Lamy, Bangui, Boukoko and Pobé to Adiopodoumé. From the last-mentioned place some parts of the Ivory Coast belonging to the coastal savannah area and farther inland were visited. The tour was made possible by means of a grant from the Netherlands Ministry of Agriculture on nomination by the Board of Governors of the State Agricultural University of Wageningen. Ample assistance and hospitality during my residence in West Africa were met with everywhere: I should like to tender my heartfelt thanks especially to the Director, Mr. Gisbourne, and Staff of the Government Agricultural Experiment Station at Samaru (North Nigeria), to the Director of the Service de l'Agriculture, Mr. Gontier, to the Director, Mr. Didelot, and Staff of the Station Expérimentale, Boukoko (Oubangui), to the Director, Mr. Rancoule, and Staff of the Station Expérimentale de l'IRHO at Pobé (Dahomey), to the Director, Prof. Mangenot, and Staff of the IDERT at Adiopodoumé, and to the Centre néerlandais, Adiopodoumé (Ivory Coast). But for the help of the said persons and institutions, it would not have been possible to bring together such an extensive collection of Leguminous material in such a short period.

On my request the Kew Herbarium in the person of Mr. Gillett undertook the revision of the herbarium material of my *Indigofera* specimens. Without the expert assistance from a systematic botanist it would not have been possible to find a way in the entanglement of modern nomenclature that is met with in the difficult genus *Indigofera*. The revised herbarium material has been inserted in the Herbarium Vadense, Laboratory of Systematic Botany, Wageningen.

The genus *Indigofera* L. is widespread in the tropical regions of both the Old and the New World. Not only a fair number of species have been spread by human aid, but non-cultivated taxa are known from all equatorial regions and also from regions situated at a slightly higher latitude.

TABLE I

Species	Collection no.	Origin	Chromosome no. 2n	n	Remarks
Subgenus A. Acanthonotus (Benth.) Benth. & Hook. f.					
1 Indigofera nummulariifolia (L.) Livera ex Alston	57043	Shika Grassland Farm (Nrth. Nigeria)	16		1. As "Indigofera echinata Willd."
Subgenus C. Indigofera L.					
Section C2. Paniculatae (Bak.) Gillett					
Subsection a. Paniculatae					
2 Indigofera paniculata Vahl ex Pers. ssp. paniculata . . .	57120	Ketou (Central Dahomey)	16		
3 id.	57124	Idigny (Central Dahomey)	16		
4 id.	57130	Ketou (Central Dahomey)	16		
5 id.	57159	Dabou (Ivory Coast)	16		
6 Indigofera paracapitata Gillett	57092	Boukoko (Oubangui)	16		6. As "Indigofera congesta Welw. ex Baker"
7 Indigofera congesta Welw. ex Baker	57093	Boukoko (Oubangui)	16		
8 Indigofera pulchra Willd.	54044 (Herb. 1954/32)	Mopoyem (Ivory Coast)	16	8	
9 id.	57005	Jos (Nrth. Nigeria)		8	
Section C3 Indigofera L.					
Subsection b. Brevi-erectae Gillett					
10 Indigofera simplicifolia Lam.	57101	Bouboko (Oubangui)	16		
Subsection d. Dissitiflorae (Bak.) Gillett					
11 Indigofera dendroides Jacq.	57125	Idigny (Central Dahomey)	16		
12 Indigofera heudelotii Benth. ex Baker var. heudelotii . .	57006	Jos (Nrth. Nigeria)	16		
13 id.	57013	Jos (Nrth. Nigeria)	16		
Subsection h. Viscosae Rydberg					
14 Indigofera secundiflora Poir.	57121	Ketou (Central Dahomey)	16		
Subsection m. Tinctoriae (Bak.) Gillett					
15 Indigofera macrophylla Schum.	57137	Abba (Central Dahomey)	16		
16 Indigofera trita L.f. subsp. subulata Ali var. subulata . .	57153	Exp. Sta. I.R.H.O., Pobé (Central Dahomey)	16		16. Seed sample 20 years old. "Sémi-rampant"
17 Indigofera arrecta Hochst. ex A. Rich	Bogor 1949	General Agric. Res. Sta. Bogor, Indonesia	16		
18 id.	57071	Samaru (Nrth. Nigeria)	16		
19 Indigofera suffruticosa Mill.	54052 (Herb. 1954/46)	Exp. Sta. CRA, Akandjé (Ivory Coast)	16		
20 id.	57155	Exp. Sta. I.R.H.O., Pobé (Central Dahomey)	16		20. Seed sample 20 years old. "Indigofera suffruticosa Mill"
21 Indigofera tinctoria L.	Bogor 1949	General Agric. Res. Sta. Bogor, Indonesia	16		21. As "Indigofera sumatrana Gaertn."
22 id.	54055 (Herb. 1954/50)	Grand Bassam (Ivory Coast)	16		
23 id.	57062	Shika (Nrth. Nigeria)	16		
24 id.	57145	Exp. Sta. I.R.H.O., Pobé, (Central Dahomey)	16		
Attributed to subsection m. Tinctoriae Bak. by Taubert in Engler & Prantl 1st edition:					
25 Indigofera dosua Buch.-Ham. ex D. Don.	Coimbra 1956	Bot. Garden, Coimbra, Portugal	48		
26 Indigofera heterantha Wall. ex Brandis	Zürich 1953	Bot. Garden, Zürich, Switzerland	48		26. As "Indigofera gerardiana Wall. ex Baker"
Subsection n. Hirsutae Rydberg					
27 Indigofera hirsuta L.	54058 (Herb. 1954/55)	Adiopodoumé (Ivory Coast)	16		
28 id.	57042	Shika Grassland Farm (Nrth. Nigeria)	16		28. Imported: origin unknown
29 id.	57123	Ketou (Central Dahomey)	16		
30 id.	57131	Ketou (Central Dahomey)	16		30. Dr. Wormer legit
31 id.	57152	Cové (Central Dahomey)	16		
32 id.	57177	Bingerville (Ivory Coast)	16		
33 Indigofera astragalina DC	57031	Shika Grassland Farm (Nrth. Nigeria)	16		33. From Plateau
Subsection p. Alternifoliolae (Harvey) Gillett					
34 Indigofera Schimperi Jaub. et Spach.	Gillett, N. Kenya 13728	Seeds received from Kew Herbarium	16		
35 Indigofera spicata Forsk	Bogor 1949	General Agric. Res. Sta. Bogor, Indonesia	32		35. As "Indigofera endecaphylla Jacq."
36 id.	54054 (Herb. 1954/49)	Azuretti (Ivory Coast)		16	36. id.
37 id.	57144	Exp. Sta. I.R.H.O., Pobé (Central Dahomey)	32		37. id.
38 id.	57150	Subgrowth under Cocos nucifera L. Semé-Podji (Dahomey)	32		38. id.
39 id.	57156	Exp. Sta. I.R.H.O., Pobé, (Central Dahomey)	32		39. id. Seed sample 20 years old, "rampant"
Section unknown					
40 Indigofera cytisoides L.	Antibes 1953	Villa Thuret, Antibes, France	48		

The use of indigo as a dyestuff is of so ancient date that it is impossible to establish the original area of several of the species which have been used for that purpose. It is a historical fact that round 2000 BC indigo dying was practised in Egypt, and it seems also to have been known in India. BURKILL (1935) supposes that the use of indigo as a dye developed independently with the Indians in America. All species signalled by Burkill for the preparation of indigo in SE Asia, come under the subgenus *Indigofera*, section *Tinctoria*. Much less data are available on the use of indigo in Africa, and no historical facts are known: the species used, however, appear to be for the greater part the same, and pertain also to the section *Tinctoria*.

In far more recent times the *Indigofera* species came to be useful in still another way: several members of both *Tinctoria* and other sections were applied as green manure, and in many tropical regions trials were started with either indigenous or imported species. Toxicity for cattle has been claimed for some of them, although the evidence on this matter is still contradictory in others (GILLETT, 1958, p. 137). The enormous variability within certain species (*I. tinctoria*, *I. hirsuta*, *I. spicata*, and others) may have been stimulated by their world-wide dispersion; thus, according to GILLETT (1958): "that the wide distribution in part by man caused obscurity as to the previous confusion of forms which is now observable". In the case of *I. spicata*, however, this remark may well be amplified by the observation that this species is a tetraploid: polyploidy in itself may be the cause of wide variation within a species.

Besides the cosmopolitical, to a certain degree cultivated, *Indigofera* species, this very extensive genus contains many representatives with a narrow area of distribution: among the latter are purely African, Asiatic, American, and Australian ones. However, quite a number have a distribution which extends over more than one continent. The monograph on the African species by GILLETT (1958) and a recent article by ALI on Pakistan and Himalayan ones (1958) clearly show the immense difficulties encountered in a number of critical cases where delimitation of species in *Indigofera* becomes necessary. Our investigations on the cytology mainly contain data from a limited number of African species: the ultimate scope being to test the eventual suitability of the cytological data as a taxonomic aid in *Indigofera*. Almost all the *Indigofera* materials discussed in this study were collected in the West African region.

Table I contains an enumeration of the *Indigofera* specimens that were studied by us, the collection number, the origin of the sample, the chromosome number, and additional notes. From by far the greater part of the specimens seedlings were obtained, and in the root tips of the latter the diploid chromosome numbers were determined. Plates 1 and 2 show the chromosome arrangements, designs of which were made at an actual magnification of 20 × 100. An attempt to ascertain the individual chromosome types in the various species met with scanty results, obviously owing to their small size. The

measuring of the entire chromosome compliments turned out to be more efficient; a list of the latter may be found in Table II.

TABLE II

Total length of mitotic chromosome complement in μ

1	Indigofera	nummulariifolia	21.4
2	,,	paniculata ssp. paniculata	38.1
3	,,	,, ,, ,,	28.5
4	,,	,, ,, ,,	32.6
5	,,	,, ,, ,,	39.1
6	,,	paracapitata	36.5
7	,,	congesta	30.0
8	,,	pulchra	30.1
10	,,	simplicifolia	28.0
11	,,	dendroides	38.9
12	,,	Heudelotii var. Heudelotii	39.1
13	,,	,, ,, ,,	32.7
14	,,	secundiflora	25.4
15	,,	macrophylla	27.3
16	,,	trita var. subulata	32.3
17	,,	arrecta	34.3
18	,,	,,	32.4
19	,,	suffruticosa	33.7
20	,,	,,	38.9
21	,,	tinctoria	35.3
22	,,	,,	26.2
23	,,	,,	26.8
24	,,	,,	29.7
25	,,	dosua	88.0
26	,,	heterantha	76.0
27	,,	hirsuta	18.8
28	,,	,,	23.8
29	,,	,,	21.2
30	,,	,,	19.4
31	,,	,,	23.8
32	,,	,,	24.7
33	,,	astragalina	23.4
34	,,	Schimperi	33.5
35	,,	spicata	45.1
37	,,	,,	49.5
38	,,	,,	50.0
39	,,	,,	53.0
40	,,	cytisoides	87.4

In both Tables I and II the species are arranged according to the systematic classification adopted in the monograph by GILLETT (1958).

Incidental information dispersed in an extensive number of publications revealed the fact that the base number in *Indigofera* may be assumed to be 8. SENN (1938) reports a number of 6 for *I. anil*, but there seems to have been a considerable confusion in nomenclature which involves the names *I. anil*, *I. tinctoria* and *I. suffruticosa*. According

to HAGERUP (1932), *I. parviflora* Heyne has a base number of 7 and *I. endecaphylla* Jacq. was reported by KISHORE (1951) as having n = 18. The available publications provides the results which are listed in Table III in alphabetical order.

TABLE III

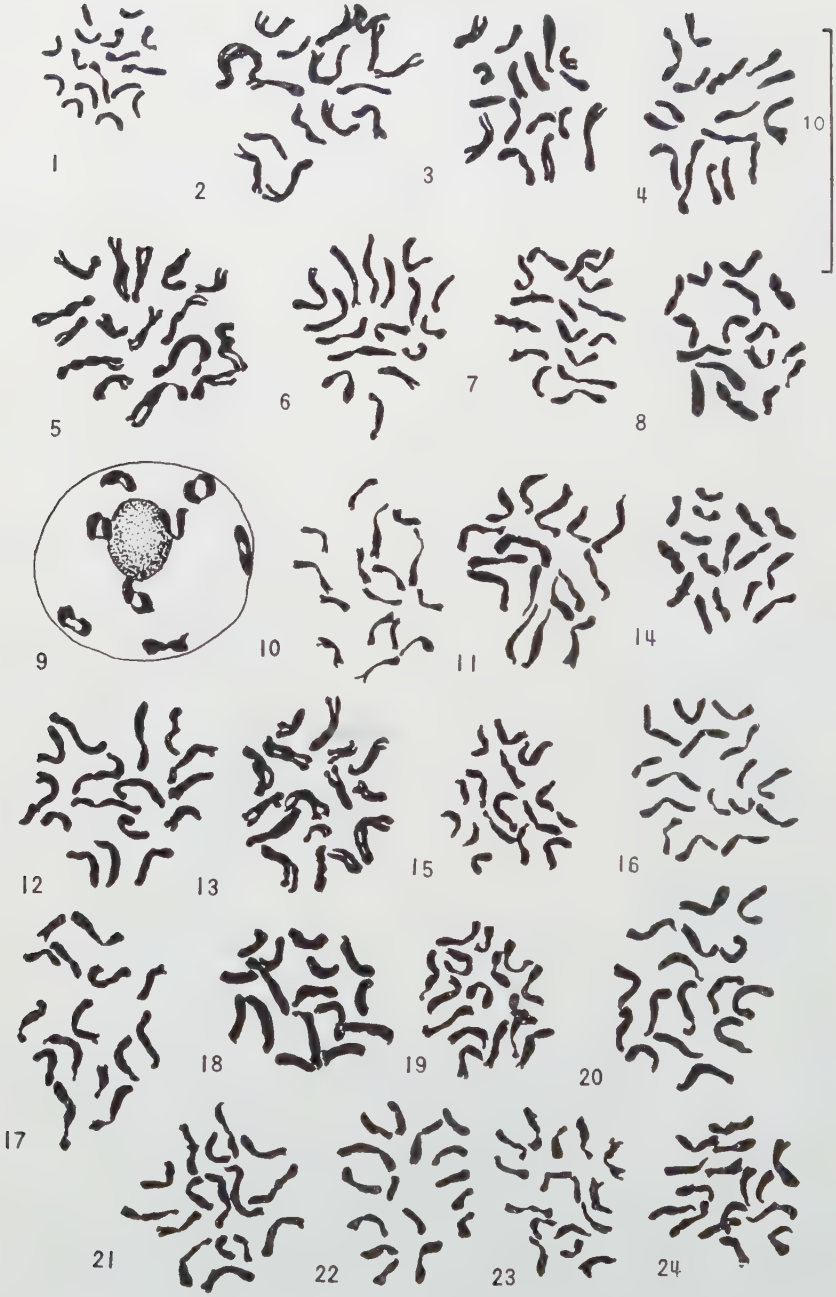
List of Indigofera species hitherto investigated

Species	n	2n	Author
I. argentea L.	8	16	Ramanathan, 1955
arrecta Hochst.		16	Frahm–L., 1953, 1957
aspera Perr.	8		Hagerup, 1932
cordifolia Heyne	8	16	Ramanathan, 1955
cytisoides L.		48	Frahm–L., 1957
diphylla Vent.	8		Hagerup, 1932
dosua Hamilt.		16	Sampath and Ramanathan, 1949
decora Lindl.		48	Tschechow, 1930
endecaphylla Jacq.	16		Frahm–L., 1953, 1957
	16		Simmonds, 1954
as: hendocephylla Jacq.	8		Turner, 1956
enneaphylla L.		16	Ramanathan, 1955
gerardiana Wall.	24		Kreuter, 1929, 1930
		48	Frahm–L., 1957
hirsuta L.		16	Frahm–L., 1953
Kirilowi Maxim.	8		Kawakami, 1930
leptocephala Nutt.	16		Turner, 1956
Lindheimeriana Scheele	8		Turner, 1956
microcalyx Bak.		32	Turner & Fearing, 1959
pseudotinctoria Matsum.	8		Kawakami, 1930
retroflexa?		16	Ramanathan, 1955
sessiliflora DC	16		Hagerup, 1932
suffruticosa Mill.	8	16	Krapovickas & Fuchs de Krapovickas, 1957
		32	Kawakami, 1930
	8		Frahm–L., 1957
sumatrana Gaertn.		16	Frahm–L., 1957
teysmanni Miq.		32	Atchison, 1951
texana Buckley	16		Turner, 1956
tinctoria L.		16	Ramanathan, 1950
trigonelloides Jaub. & Spach.	8	16	Ramanathan, 1955
viscosa Lam.	8		Hagerup, 1932

It is to be regretted that many of these reports have been based upon counts in either metaphase I or II of the reduction division (HAGERUP, 1932, KAWAKAMI, 1930, KISHORE, 1951, KREUTER, 1930, RAMANATHAN, 1950, 1955, SAMPATH and RAMANATHAN, 1949, SENN, 1938, SIMMONDS, 1954), and that illustrations are sometimes lacking, for in the course of our studies it appeared that there exists a considerable difference in chromosome dimensions between the various species (Plates 1 and 2).

When summing up the lines of investigation along which it seems possible to discern the various chromosome patterns hitherto found in *Indigofera* we come to the following results.

1° Apart from a few devious reports, there are three main chromosome groups, viz. 2n = 16, 2n = 32 and 2n = 48.



Plates 1 and 2. The numbers of the chromosome complements correspond to the numbers of the species listed in Table I. All the designs were made from metaphase plates in root-tip cells, except for the PMC diakinese figures 9 and 36. The unit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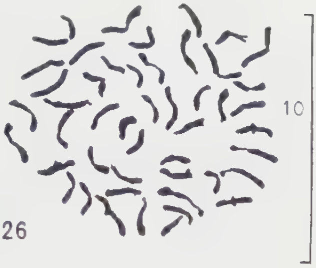

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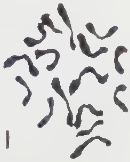
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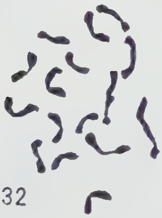
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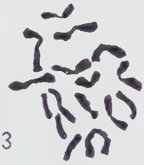
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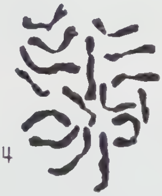
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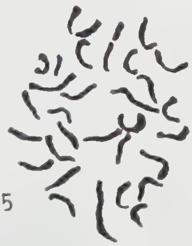
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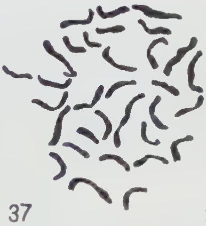
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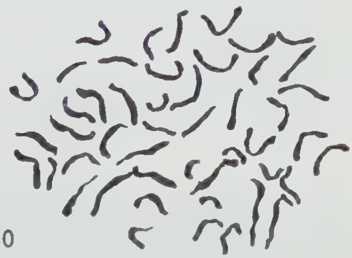
40

of magnification is 10 μ. The drawings were made from sectioned material previously fixed in Navashin fluid and subsequently stained with crystal violet.

2° In the 2n = 16 group there are types with rather slender chromosomes (*I. nummulariifolia*, *I. simplicifolia*), with short and thick chromosomes (*I. secundiflora*), with short chromosomes (*I. hirsuta* and *I. astragalina*); the others have rather long and relatively stoutish chromosomes.

3° We mentioned the fact that discernment of individual chromosomes met with difficulties and confusion owing to the small size. The total length of the complements, however, provides sufficient results to justify further investigation. The material which has hitherto been studied (Table II) in this way, however, is too scanty to allow far-reaching conclusions.

4° It may be pointed out that most of the types studied by us tend to be perennial and to become more or less shrubby and woody. The small-chromosome types *I. nummularifolia*, *I. hirsuta* and *I. astragalina*, however, are distinctly herbaceous; this, more or less, also holds for *I. secundiflora* and certainly for the tetraploid *I. spicata*.

5° The 32-chromosome species hitherto reported, are either Afro-Asiatic (*I. sessiliflora*), American (*I. leptocephala*, *I. texana*), Asiatic (*I. teysmanni*), or pantropical (*I. spicata*). The only endemic African species belonging to this group is *I. microcalyx*. All other typical African species with endemic distribution that were studied up till now, have 2n = 16.

As to the—perhaps—endemic species *I. teysmanni*, this plant has been included by ATCHISON in her report on chromosome numbers of Leguminous woody species (1951), as a tree. The original description by MIQUEL in his "Flora van Nederlandsch-Indië" (1855) says that it is a shrub from the shores near Siboga (North Sumatra): "Erecta, fruticosa——", etc. A near relative is *I. galegoides* DC, a shrub 1–2.50 m high, not uncommon in light woody undergrowth. The genus *Indigofera* does not include real trees.

6° The 48-chromosome species *I. dosua*, *I. heterantha* and *I. cytisoides* are of Himalayan origin. This group, certainly, deserves a deeper study.

SUMMARY

The West African species of *Indigofera* investigated in this study reveal n = 8 to be the common basic number.

Whereas identification of the individual chromosomes met with no success, a distinct difference in dimensions could be detected in a number of cases, and also definite differences in the total length of the chromosome complements.

The endemic African species are, with one exception, diploid. Tetraploidy occurs in Afro-Asiatic, Asiatic, American and pantropical species, whereas hexaploidy has been found in some Himalayan species.

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ÜBER EINE 2N – 4N SEKTORIALCHIMÄRE BEI OENOTHERA

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(*Amsterdam*)

(*received June 5th, 1960*)

Das Studium von GEITLER's Buch über Endomitose erinnert mich an eine bis jetzt noch nicht veröffentlichte Beobachtung aus den letzten Jahren meiner Arbeit im hiesigen Versuchsgarten über das Auftreten einer 2n – 4n Sektorialchimäre bei einer *Oenothera*. Eine kurze Erwähnung ist hier vielleicht noch am Platze.

Nachdem BOEDIJN (1924) die *O. Bauri*, in der Literatur auch *O. hungarica* Borb. genannt, beschrieben hatte, habe ich diese Art viele Jahre weiter gezüchtet. Im Jahre 1928 kreuzte ich die *O. Lamarckiana* mit der *O. Bauri*. Daraus erhielt ich im folgenden Jahre 58 Pflanzen, von denen 50 vom Velutina-Habitus nach der Terminologie von HUGO DE VRIES, 8 vom Laeta-Typus waren, ein Resultat, das gut übereinstimmte mit dem von Frl. BAERECKE in 1944 beschriebenen. Sie erhielt aus einer Kreuzung 65 Pflanzen, von denen 57 vom Typus velans undans, 8 vom Typus gaudens undans waren, wie man gegenwärtig auch sagen kann (Bekanntlich hat die *O. Lamarckiana* gaudens- und velans-Geschlechtszellen, die *O. Bauri* laxans-Eizellen und undans-Pollenkörner). Auf eine nähere Beschreibung der beiden Typen kann ich hier verzichten, da sie von Frl. Baerecke gegeben wurde. Von beiden wurde nun ein Exemplar geselbstet. Die Velutina-Pflanze lieferte eine reichliche, konstante Nachkommenschaft, die keine Spur der Hemikleistogamie der *O. Bauri* mehr zeigte. Von der Laeta-Pflanze erhielt ich aber nur zwei Nachkommen, die zudem noch etwas gescheckte Blätter zeigten, aber sonst den elterlichen Typus wiederholten. Die weitere Nachkommenschaft brachte ein paar Überraschungen. Im Jahre 1931 erhielt ich nur 6 Keimpflanzen, in 1932 nur 5, aber in 1933 war die Keimung plötzlich reichlich und hatte ich eine Kultur von 60 Pflanzen. Ein paar Jahre später, in 1936, war die Keimung wieder einmal schlecht und konnte ich nur 10 Pflanzen groszziehen. Die Kultur von Jahr zu Jahr fortsetzend, machte ich in 1941 die uns hier interessierende Beobachtung. Bei einer Pflanze entdeckte ich einige viel zu dicke Blütenknospen, die an Gigas-Knospen erinnerten. Bei genauerem Zusehen stellte sich heraus, dasz ein ganzer Sektor der Infloreszenz tetraploid sein muszte, sogar ein in diesem Sektor entspringender Seitenzweig. Den Beweis lieferten die Pollenkörner, die fast durchweg vierlappig waren, wie bei jeder Gigas-Rasse; auch die Nachkommenschaft dreier geselbsteter Blüten, die mir 17 Pflanzen lieferten, welche sich weigerten, im ersten Jahr zu blühen, wie man das bei Gigas-Rassen gewohnt ist, und weiterhin zu einer konstanten Gigas-Rasse unserer *O. Lamarckiana* × *Bauri laeta* führten.

Erwähnt sei hier noch, dass eine Gigas-Pflanze sich im Jahre 1943 durch eine auffallend dichte Behaarung der Knospen von den anderen Pflanzen unterschied. Auch sie wurde geselbstet, aber bei den 30 Nachkommen war von einer solchen Behaarung nichts mehr zu sehen.

Wie unsere 2n – 4n Sektorialchimäre genau entstanden ist, lässt sich schwer sagen. Es kann natürlich in der Initialzelle des tetraploiden Sektors eine Endomitose stattgefunden haben, aber auch eine Verschmelzung zweier Kernanlagen während der Telophase. Übrigens ist es nicht das erste Mal, dass bei *Oenothera* eine chromosomale Chimäre entdeckt wurde. Schon in 1928 beschrieb SCHWEMMLE eine teils diploide, teils tetraploide Pflanze von der F_2 des Bastards *O. Berteriana* × *odorata* und in Flora 133, S. 220, 1939 bildete RENNER eine solche von *O. atrovirens* × *biennis* ab.

Wie Frl. Baerecke führte ich auch die reziproke Kreuzung *Bauri* × *Lamarckiana* aus. Ich erhielt 58 blühende Pflanzen, von denen 57 vom Typus laxans velans waren, und nur eine Pflanze vom Typus laxans gaudens. Unsere Beschreibungen der beiden Typen stimmen gut überein, nur waren die Blüten bei mir etwas kleiner und für die laxans velans Pflanzen notierte ich, dass die Blätter gedreht waren wie bei der *O. Bauri*. Da auch diese beiden Typen sich als konstant erwiesen, erhielt ich also aus den Kreuzungen zwischen *O. Bauri* und *O. Lamarckiana* nicht weniger als 5 konstante Bastarde.

Anhangsweise sei nun noch etwas über weitere Kreuzungen mitgeteilt. Ich kreuzte z.B. die Kombinationen gaudens undans und velans undans aus *O. Lamarckiana* × *Bauri* zurück mit der *O. Lamarckiana*. Als Resultat erhielt ich 30 resp. 36 *Lamarckiana*-Pflanzen, die nur Unterschiede in der Blütengrösze zeigten. Dagegen ergab die Kreuzung der laxilaeta (aus *O. Bauri* × *Lamarckiana*) mit der *O. Bauri* eine Spaltung meiner 30 Pflanzen in 14 typische kleistogame *Bauri*-Individuen und 16 laeta-Pflanzen.

Wie Frl. Baerecke führte ich auch die reziproken Kreuzungen zwischen *O. Bauri* und der Mutation *blandina* der *O. Lamarckiana* aus. Der Bastard *O. Bauri* × *blandina* sah bei mir der Kombination laxans velans vollkommen ähnlich und blieb weiterhin konstant, abgesehen von einer Spaltung nach der Blütengrösze. Ich bestäubte ihn noch mit der *O. Bauri* und beobachtete auch hier eine Spaltung: von 20 blühenden Pflanzen waren 6 *Bauri*, 14 eher vom *blandina*-Typ. Die F_2 des Bastards *O. blandina* × *Bauri* schien mir nicht einheitlich. Von 25 blühenden Pflanzen erinnerten 8 an *O. Bauri*, 17 an *O. blandina*. Die Rückkreuzung einer F_1-Pflanze mit der *O. blandina* ergab nur wieder *O. blandina*. Die Eizellen der F_1-Pflanzen trugen also *blandina*-Charakter.

Die reziproken Kreuzungen zwischen *O. Bauri* und *O. biennis* beantworteten der Erwartung. Bekanntlich hat letztere Art albicans-Eizellen und rubens-Pollenkörner. Die Verbindung *O. Bauri* × *biennis* lieferte mir 55 uniform blühende Pflanzen mit breiten Blättern, wie die der *O. biennis*, die aber gedreht waren, wie bei der *O. Bauri*, und mit zahlreichen kleistogamen Blüten. Die Nachkommenschaft blieb konstant und eine Kreuzung mit der *O. Bauri* lieferte nur wieder

Bauri. Die reziproke Kreuzung *O. biennis* × *Bauri* ergab 60 Pflanzen, die eher an *O. Bauri* erinnerten, mit schmaleren gedrehten Blättern. Auch hier blieb die Nachkommenschaft konstant und die Rückkreuzung mit *O. biennis* lieferte nur *biennis*.

Die Kreuzungen zwischen *O. Bauri* und der *O. muricata* unserer Dünen gelangen nur zum Teil. Die heterogame *O. muricata*, in der Literatur auch *O. syrticola* genannt, hat rigens-Eizellen und curvans-Pollenkörner. Die Kombination laxans curvans aus der Verbindung *O. Bauri* × *muricata* brachte es bei mir nicht weiter als sterbende Keimlinge, im Gegensatz zu dem, was Frl. Baerecke dafür mittteilte. Die reziproke Kreuzung *O. muricata* × *Bauri* aber ergab eine reiche F_1, uniform mit nahezu ungetupften Stengeln, schmalen, etwa 4 cm breiten Blättern, nur 1.7 cm langen Kronblättern und ohne kleistogame Blüten. Die F_2 war aber nicht mehr so schön uniform. Von 51 blühenden Pflanzen waren 33 kräftig vom *Bauri*-Typ, 18 schwächer, und bei beiden Typen kamen jetzt wieder viele kleistogame Blüten vor. Bei den schwachen Pflanzen beobachtete ich auch apetale Blumen oder solche mit einem Kronblatt. Die Rückkreuzung einer F_1-Pflanze mit der *O. muricata* hatte auch schlechten Erfolg. Die meisten Keimlinge starben aus Mangel an Chlorophyll früh ab und es gelang mir nur, 3 Pflanzen grosz zu ziehen, die der *O. muricata*, wie erwartet, ähnlich waren.

Zuletzt dann noch etwas über Kreuzungen der *O. Bauri* mit der isogamen *O. Hookeri*. Aus der Verbindung *Bauri* × *Hookeri* erhielt ich 30 Pflanzen, uniform, Ende August über 2 M hoch, mit dunkelroten fein getupften Stengeln, weisznervigen *Hcokeri*-artigen, jedoch etwas gedrehten Blättern und kleinen Blüten. Die F_2 blieb konstant, aber 7 von 30 Pflanzen hatten jetzt grosze Blüten. Die Rückkreuzung mit der *O. Bauri* lieferte nur wieder *Bauri*, aber ohne Andeutung auf Kleistogamie. Meine F_1 *Hookeri* × *Bauri* umfasste 60 Pflanzen, die durch die rote Färbung und Rotpunktierung der Stengel und die stark gewellten rotnervigen Blätter der *O. Bauri* ähnlich sahen, aber ziemlich grosze Blüten mit einem Diameter von 5 cm hatten. Eine Selbstung führte zu einer F_2 von 24 Pflanzen, von denen 5 eher an den F_1-Bastard erinnerten, 19 an *O. Hookeri* mit meistens zu kleinen Blüten. Die Schlussfolgerung, dasz der F_1-Bastard *Hookeri*-Eizellen und *Hookeri* + *undans*-Pollenkörner hatte, wurde bestätigt durch eine Rückkreuzung mit der *O. Hookeri*, die nur *Hookeri*-artige Pflanzen lieferte, diese aber zum Teil mit groszen, zum Teil mit kleinen Blüten.

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NOTES ON THE PTERIDOPHYTES OF THE DUTCH WEST INDIES

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(*received May 9th, 1960*)

The present paper is intended as an introduction to the author's treatment of the ferns and fern allies in Mr. A. L. Stoffers' new Flora of the Netherlands Antilles, the first volume to be published shortly. Apart from phytogeographical notes that do not usually find a place in such a flora, in the following some nomenclatural notes are given, besides a few explanatory remarks on the principles employed in the classification.

I. Taxonomic notes

A. The system

In connection with the increased interest in fern taxonomy of the last decades several systems of classification have been proposed which, especially in the Leptosporangiate ferns, show no small degree of diversity. Though most of these systems contain definite elements of advance, it is felt that none of them has been sufficiently worked out in every respect to be acceptable as a whole. (The interested reader may find a review of these systems in Pichi–Sermolli, 1959). Consequently none of these systems has been entirely adopted for the treatment under consideration. With regard to the family treatment the author has been rather conservative and has maintained the *Polypodiaceae* in their old, inclusive circumscription; not because he believes that they are a natural entity, but because it seems to him that none of the various ways in which they have been subdivided is quite satisfactory.

On the generic level a more modern concept has been adopted. With regard to the genera recognized the writer finds himself in rather close agreeance with Proctor (1953). The Thelypteridoids, for instance, are treated as distinct from the Dryopteridoids, and *Cyclosorus*, *Goniopteris*, *Meniscium*, etc., have been united with *Thelypteris* (see also Morton, 1950). It seems that the differences between the Grammitidoid and the Polypodioid ferns have also been sufficiently demonstrated; but the author prefers to recognize at least provisionally the genus *Xiphopteris*, rather than uniting all Grammitidoids under *Grammitis* (Proctor, l.c.). The splitting of *Hymenophyllum* (Copeland, 1938, 1947) is, in the writer's opinion, an unnatural procedure. *Trichomanes*, *Polypodium* (exclusive of the Grammitidoids formerly referred to it), and *Lycopodium* are perhaps artificial genera when treated in their old, very wide circumscription, but too many segregates have been proposed, and before a general revision of the species

is undertaken they can better stand as they are, in an inclusive sense. In the same way the genus *Cyathea* has been treated; the "single character" genera *Alsophila* and *Hemitelia* are, it is believed, justly rejected by most contemporary pteridologists, as long as no associated characters are found.

The sequence of genera in the *Polypodiaceae* is such that related genera are found side by side; where opinions disagree such resemblances have also been taken into account that spring to the eye but may eventually be shown to be without a natural basis.

Apart from the citations of the original descriptions of the species or transfers of the combinations only such papers have been cited that are of major importance for the floristics of the West Indian Pteridophytes or that contain more or less monographic treatments.

B. Nomenclatural notes

Xiphopteris serrulata (Swartz) Kaulfuss

This combination is usually cited as Kaulfuss, Enum. Fil. 85. 1824, for instance by COPELAND (1952). In the indicated place Kaulfuss mentions the genus, but *Grammitis serrulata* Swartz is only listed as one of the constituent species, without actual transfer of the name. The valid combination is, however, found in the index (p. 300). The basionym is *Acrostichum serrulatum* Swartz, Prodr. 128. 1788.

Thelypteris tetragona (Swartz) Small **var. guadalupensis** (Fée) Kramer, comb. nov.

Basionym: *Goniopteris guadalupensis* Fée, 11e Mém. 64. 1866.

This combination, previously published under *Polypodium* and *Dryopteris*, is new under *Thelypteris*.

Blechnum striatum (Swartz) C. Christensen, Index Filicum 160. 1906 (basionym: *Onoclea striata* Swartz, Syn. Fil. 304. 1806); not of R. Brown, Prodr. Fl. N. Holl. 152. 1810.

This is one of several cases in CHRISTENSEN's Index Filicum where a name is adopted in spite of the presence of an earlier homonym, only because its basionym is oldest. The same work lists two synonyms of the present species, *Lomaria tuberculata* J. Smith and (in the Corrigenda of the first supplement) *Lomaria ryani* Kaulfuss. BROADHURST (1912) in addition listed *Lomaria brasiliensis* Raddi as a doubtful synonym. The last name is, of course, not available under *Blechnum* because of *Blechnum brasiliense* Desvaux; it is also very doubtful whether it applies to the same species. The name *Lomaria tuberculata*, as I was very kindly informed by Mr. F. Ballard, Kew, was not published with a description, nor could a type specimen be found in the herbarium of the British Museum. The type of *Lomaria ryani* Kaulfuss in the same herbarium was examined for me by Mr. W. Punt, Utrecht, to whom I am much indebted for this service. As was already suspected after a scrutiny of the description, it differs in two important characters from the species under consideration, namely, the strongly falcate

pinnae and the pubescence of the costae, especially on the adaxial side. The name *L. ryani* applies therefore to another species. Since the author does not recognize the genus *Struthiopteris*, under which the epithet *striata* could be used, as distinct from *Blechnum*, a new name is required:

Blechnum nesioticum Kramer, nom. nov.

Type (the type of *Onoclea striata* Swartz) from St. Christopher (St. Kitts) in herb. S-PA!

II. Phytogeographical remarks

As will be seen from the taxonomic treatment, not only a certain number of species hitherto unrecorded from the Dutch West Indies are included, but many amendments had to be made to Boldingh's accounts (1909, 1913), with respect to the nomenclature employed as well as in terms of corrections of identifications. There is no need to go into this subject in detail, as the notes on synonymy will provide sufficient information.

At present 74 native species of Pteridophytes in 32 genera are known from the Dutch West Indies. As the number of species found in the "Leeward Group" (Aruba, Curaçao, Bonaire) is very small, only 8 species in 8 genera, they will not further concern us here. It may be remarked in passing that in the Dutch Islands the *Schizaeaceae* (*Anemia* and *Lygodium* with one species each) and the *Marsileaceae* (one species of *Marsilea*) are confined to the Leeward Group; the last-named represents the only case of near-endemism, *Marsilea ernestii* A. Braun being apparently only known from Bonaire and the vicinity of Caracas, Venezuela.

The "Windward Group" (Saba, St. Eustatius, St. Martin) possesses 70 species in 28 genera. [1]) It is somewhat surprising that the smallest island, Saba, has the largest number: 57 species in 22 genera, whereas St. Eustatius has 32 species in 18 genera, and the largest island, St. Martin, only 15 species in 8 genera. This is, however, readily explained by the physiography of the islands. Saba reaches the highest elevation (900 m), and a number of species are confined to its highest peak, the Mountain. Thus tiny Saba (12 km²) in terms of the fern flora compares quite favourably with the neighbouring but much larger and also loftier island of St. Kitts (ca. 160 km², over 1300 m), which according to Box & Alston (1937) has 97 species of Pteridophytes. Dominica, on the other hand, in the group of oldest and highest islands of the Lesser Antilles, with ca. 730 km² and an elevation of up to ca. 1450 m, has a Pteridophyte flora of nearly 200 species (Hodge, 1954).

The genera with the largest number of species are *Polypodium* (11), *Thelypteris* (8), *Asplenium* (6), *Elaphoglossum* and *Lycopodium* (4), *Cyathea* (sens. lat.), *Hymenophyllum*, *Selaginella*, and *Pteris* (3). *Cyathea* is, strangely enough, almost restricted to Saba; *C. arborea* occurs also on St. Eustatius but seems to be quite rare. The three species of tree-ferns

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[1]) Varieties are disregarded in these and the following figures.

of the Dutch islands are the same as those found on St. Kitts. *Hymenophyllum* and *Selaginella* are confined to Saba; *Trichomanes* and *Lycopodium* are also present on St. Eustatius, each with one species which is absent from Saba.

Floristically the most interesting species of the Dutch islands is probably *Lycopodium wilsonii* Underw. & Lloyd, described from Puerto Rico, later reported from Guadeloupe and Colombia, and very recently collected in Suriname; some fragments were found among a rather old collection of *L. setaceum* from Saba.

While the physiography of the non-volcanic island St. Martin is unfavourable to the growth of ferns, the volcanoes of Saba and St. Eustatius possess plant communities where a number of epiphytic and terrestrial species flourish. Yet it is surprising that in spite of their proximity there is such a difference in their fern flora, though the larger number of species on Saba is explained by its higher elevation. Thus it is difficult to understand why the widespread species *Polypodium pectinatum* occurs on St. Eustatius but on Saba is replaced by its equally widespread relative *P. plumula*; why Saba has *Lycopodium taxifolium* (as well as two other species), St. Eustatius only *L. dichotomum*, etc. In some instances incompleteness of our knowledge of the flora may account for the seemingly random distribution of species over these islands; but collecting has been relatively quite intense on these small islands and this explanation hardly holds good in all cases. Likewise, the absence of other widespread species that occur on many adjacent islands, such as *Hypolepis repens* and *Lycopodium cernuum*, is rather astonishing.

When the 70 species of the Windward Group are arranged according to their pattern of general distribution, the following figures are obtained:

Tropics of the New and at least parts of the Old World	9	species
Throughout the American Tropics (sometimes also Subtropics)	29	,,
Circum-Caribbean (i.e., Greater and Lesser Antilles, Central and South America, sometimes also Florida)	9	,,
Antilles and Central America	2	,,
Antilles and South America	5	,,
Antilles and Florida	2	,,
Antilles only [1])	9	,,
Lesser Antilles only	5	,,
	70	,,

Species with a very wide distribution form more than one-half of the fern flora which therefore can be said to show a lack of peculiarity. Among the species with a more limited area those with an Antillean distribution are relatively more numerous on Saba (11 out of 57) than on St. Eustatius (4 out of 32); species with a Caribbean distribution (West Indies and adjacent parts of Central and/or South America) are equally distributed over both islands (16 out of 57 on Saba, 9 out of 32 on St. Eustatius, that is approximately 2/7 in

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[2]) Mainly in the *Hymenophyllaceae*, *Selaginella*, and *Elaphoglossum*.

both cases). For St. Martin the absolute numbers are too low for such calculations. Likewise it is impossible to conclude from the distributional patterns whether the floristic influence from the South (Lesser Antilles, South America) or from the West (Greater Antilles, Florida, Central America) is strongest, though this may be possible when the whole flora of the islands is taken into account, as was done to a certain degree by STOFFERS (1956). The matter is of interest in connection with the problem whether the Lesser Antilles are to be regarded as oceanic islands or not (see CHRYSLER, 1948, and BEARD, 1949).

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NEW SPECIES OF TARAXACUM FROM ASIA
(INCLUDING: RECHINGERI, ITER IRANICUM II Nr. 40)

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('s Gravenhage)

(received June 19th, 1960)

This paper mainly is based on rather large collections of *Taraxacum* for revision send to me by P. Aellen and K. H. Rechinger, collected for a great deal in Iran; some material from other collections is used in addition. This paper only deals with new forms. In both collections I, furthermore, noticed the following species: *T. assemani* Boiss., *T. primigenium* H.M. sens. str., *T. fulvipile* Harvey, *T. haussknechtii* Uechtr., *T. leucanthemum* Ledeb., *T. luridum* Hagl., *T. aleppicum* Dahlst., *T. fedtschenkoi* H.M., *T. monochlamydeum* H.M. em. Hagl., *T. wallichii* De C., *T. brevirostre* H.M., *T. parvulum* De C., *T. kurdicum* H.M. ex Nábělek, *T. stevenii* (Spreng.) De C., *T. protractifolium* Hagl., *T. pseudonigricans* H.M., *T. puberulum* Hagl., *T. calocephalum* H.M. em. Dahlst., *T. montanum* (C.A.M.) De C., *T. syriacum* Boiss. Especially *T. fulvipile*, *T. haussknechtii* and *T. syriacum* are very abundant in these collections; according to von Handel–Mazzetti (1907) and Rechinger (1959), *T. haussknechtii* was only known from the Balkan Peninsula! and Libanon.

Most of the new-described species belong to already known sections; one new section (*Macrocornuta*) is described; in future, probably two new sections have to be set up in order to gain more systematical insight into the complexity of the genus in the Orient.

Taraxacum iranicum van Soest **spec. nov.** (Fig. 1)

Planta mediocris—parva 3–7 cm alta araneoso-pilosa, basi fragmentis foliorum vetustorum incrassata.

Folia juniora araneosa denique glabrescentia, lanceolata ad 8 cm longa petiolo breve purpureo inclusa, nervo mediano praesertim parte inferiore purpureo; folia exteriora canescenti-viridia lobata, lobi laterales (utrinque ca. 4) triangulares vel deltoidei vel falcati, summo acuto retroverso, dorso convexo magne dentati, margine inferiore concavo saepe integro, interlobiis angustis saepe dentatis, interdum sublobatis, lobus terminalis ad 2 cm longus hastatus vel subsagittatus interdum indeterminato-sublobatus, dentatus vel denticulatus, subacutus.

Scapi 1–5 subcrassi (sub involucro dense) araneosi.

Involucrum mediocre 13 mm longum ad 11 mm latum glaucoviride. Squamae exteriores lanceolatae vel oblanceolatae, ad 7 mm longae ad 2,5 mm latae laxe adpressae apice recurvatae, late albomarginatae apice pro parte inconspicue callosae; squamae interiores aequilatae, apice pro parte callosae.

Calathium planum radians ad 2,8 cm diametro flavum(?); ligulae marginales planae extus stria pallide purpurea vel roseola notatae. Antherae polliniferae; stylus et stigmata subobscura. Floret aestate.

Achenium olivaceo-brunneum subcylindricum ad 1 mm latum 5,5 mm longum (pyramide inclusa) superne brevissime spinulosum ceterum subrugosum—basi laeve, in pyramiden conico-cylindricam ca. 1 mm longam spinulis saepe praeditam sensim abiens. Rostrum ca. 4 mm longum; pappus albus 6 mm longus.

TYPUS: Iran, Khorasan: Montes Hazar Masdjid ca. 2500 m, 8–9.6.1948; K.H. et F. Rechinger, It. Iran. II, 1948, Plantae P. Aellen coll. lect., no. 5061.

On the same spot, no. 5090, together with *T. oliganthum* Schott & Kottschy and *T. haussknechtii* Uechtr.

Furthermore:

Iran, Gorgan: in declivibus borealibus montium Shavar pr. Hadjilang 2400–2600 m, 26–27.7 1948, K.H. et Rechinger, It. Iran II, 1948.

Iran, Damghan-Semnan: Zentral-Elburs, Berg ca. 8 km nördlich Djaschm, Felsschutt 2900 m, 29.7.1948 E. Behboudi & P. Aellen (h), together with *T. oliganthum* Schott & Kottschy.

This species seems to be very allied to *T. oliganthum* Schott & Kottschy and *T. brevirostre* H.M.; the first one has somewhat narrower outer involucral bracts, a slightly coloured pappus, different leaves; the second one has a thicker rostrum; both are generally much more hairy.

But also *T. iranicum* in its youth is provided with a short hairy cover. The leaves are reminescent to those of *T. pseudo-nigricans* H.M., but this species generally has more developed horns on the involucral bracts, and, especially, its achenes are much more spinulate.

VON HANDEL–MAZZETTI (1907) classed *T. oliganthum* into sect. *Rhodotricha* H.M., because of its coloured pappus, but the colour is very faint. Also that of *T. brevirostre* is not purely white and this species is classed by him in sect. *Parvula* H.M., with which I can not agree. I believe *T. oliganthum*, *T. brevirostre* and *T. iranicum* together belong to a distinct and new section, of which I, however, prefer to postpone its description.

Taraxacum rechingeri van Soest **spec. nov.** (Fig. 2)

Planta 5–12 cm alta parce araneosa.

Folia ad 15 cm longa petiolo saturate purpureo inclusa, nervo mediano praesertim parte inferiore purpureo. Lobi laterales (utrinque ca. 3–4) breves ad 1,2 cm longi triangulares valde retroversi acuti integres vel raro dorso 1 dente minuti. Interlobiis angustis ad 1 cm longis interdum denticulatis vel parve dentatis. Lobus terminalis elongatus subsagittatus apice ± rotundatus, lobuli basis subacuti, lobulo apicali interdum inciso.

Scapi floriferi foliis breviori, paulo araneosi, sub involucrum glabri.

Involucrum ca. 12 mm longum ca. 13 mm latum atroviride.

Squamae exteriores laxe adpressae ovatae ad 7 mm longae conspicue anguste albo-marginatae vel roseolo-marginatae ciliolatae laeves summo elongato obtuso. Squamae interiores 2,5–3 mm latae membranaceo-marginatae laeves.

Calathium planum radians ad 2,5 cm diametro pallide luteum. Ligulae marginales planae extus stria cano-violacea notatae. Antherae polliniferae. Stylus et stigmata virescentia. Floret vere.

Achenium ignotum, pappus albus.

TYPUS: Iran, Teheran: Montes Elburs centr., in declivibus australis montis Točal inter Darband et Pasgaleh, 1400–1600 m, 8.4.1948; K.H. et F. Rechinger, It. Iran. II, 1948, no. 2632; also 2627.

This species does not seem to be nearly related to any known form of *Taraxacum*; in some respects it may be related to *T. stevenii* (Spreng.) De C., which easily can be distinguished by blackish styles and by nearly integer, dentate leaves; in other respects, especially in relation to the involucre, it reminds of *T. scaturiginosum* Hagl. (from Greece into the Orient up to Kurdistan), but also the leaves of this species are quite different. As ripe achenes are lacking, it is impossible to fit this species at this moment into a section.

Sectio Macrocornuta van Soest sect. nov.

Achenia parva ca. 3,5 mm (pyramide inclusa), brunnescentia vel straminea, rostrata, pappus niveus. Involucrum in stato juvenilis semicoronatum ut squamae interiores cornis angustis vel corniculis magnis instructae sunt. Calathium parvum flavum.

TYPUS: *T. wallichii* De Candolle (1838).

The section consists, as far as known nowadays, of the following species:

1. *T. wallichii* De C. in its strict sense, not in the large sense as treated by VON HANDEL–MAZZETTI (1907), who gave the following distribution: "*in aridis siccis a regione Caspica et Syria usque ad Indiam et Mongoliam*". I only know *T. wallichii* from Iran, Afghanistan and the western Himalayas in Pakistan and India. The material from Syria belongs to *T. aleppicum* Dahlst., belonging to sect. *Scariosa* H.M. em. Dahlst. (1926).
2. *T. monochlamydeum* Handel–Mazzetti em. Hagl. (1938); Haglund mentioned: Turkestan!, China (Kansu!, Sinkiang!) and Mongolia!; I can add: Afghanistan and Ladak.
3. *T. afghanicum* m. from Afghanistan.
4. *T. neolobulatum* m. from Iran, Beluchistan and Kulu Himalaya; both latter species are discussed below.

The section *Scariosa* H.M. (1907), very heterogeneous, has been already discussed by Dahlstedt, who restricted it to *T. bithynicum* De C., *T. aleppicum* Dahlst, and a series of species belonging to the very complex species: *T. megalorrhizon* (Forskål) H.M.; the area of

this group covers the Mediterranean region, including the African and Asian coastal districts along this sea. Moreover, von Handel–Mazzetti's section contained *T. obovatum* (Willd.) De C., which, together with a few allied species, belongs to sect. *Obovatum* m. (1954); next, it contained the forms, now enclosed in sect. *Macrocornuta* and finally *T. glaucanthum* (Ledeb.) De C., which seems to be nearly related to sect. *Eu-Erythrocarpa* Dahlst. (see below).

Taraxacum afghanicum van Soest **spec. nov.** (Fig. 3)

Planta mediocris ca. 10 cm alta araneoso-pilosa.

Folia numerosa decumbentia ad 15 cm longa lobata paulo canescentia, interdum irregulariter purpureo-maculata, petiolis subangustis nervoque mediano purpureo-colorata. Lobi laterales (utrinque ad 6) triangulares ca. 1 cm longi dorso dentato summo retroverso acuto, interlobiis ad 7 mm latis valde dentatis vel sublobatis; lobus terminalis sagittatus ad 2 cm longus ± denticulatus vel interdum dentatus, subacutus vel subobtusus.

Scapi (sub involucro dense) araneosi.

Involucrum ad 13 mm longum ad 11 mm latum; squamae exteriores ad 5 mm longae apice recurvatae ovato-lanceolatae ± purpurascentes callosae—obsolete corniculatae, interiores cornutae vel corniculatae.

Calathium planum ad 2 cm diametro flavum. Ligulae marginales planae extus stria cano-violacea notatae. Antherae polliniferae; stylus et stigmata clare lutea. Floret vere.

Achenium stramineum parvum ad 3,5 mm longum (pyramide inclusa) costulatum superne spinulis subcrassis brevis praeditum, ceterum rugosum, in pyramiden cylindricam 0,5 mm longam costulatam spinulis saepe praeditam subabrupte abiens; rostrum 7 mm longum, pappus niveus 6 mm longus.

E sectione Macrocornutorum van Soest.

Typus: Afghanistan: Jalalabad valley, L. Edelberg; 3rd Danish Exp. to Central Asia no. 20, 20.2.1948 (h.W).

Also: Afghanistan, Griffith no. 3359 (h.K., pro parte); Afghanistan, Sarobi, Grabenränder, 17.3.1952 Volk no. 2468 (h.W).

This species is easily distinguished from the other species of this section by the shape of the leaves; *T. neo-lobulatum* has side lobes of the leaves perpendicular to the median nerve, not recurved; *T. wallichii* has extremely divided leaves. *T. monochlamydeum*, likewise with a different leaf form, is distinguished by a clear or often faint pink colour of the stripes on the outside of the ligulas, by less purple petioles of the leaves, less spinulate achenes and by often strongly cornute outer involucral bracts.

Taraxacum neo-lobulatum van Soest **nom. nov.**; *T. lobulatum* Bornmüller 1892 nomen nudum, Dahlstedt 1926, cum icones, non *T. lobulatum* Brenner 1907.

The description, given by Dahlstedt, is quite sufficient. This species is allied to *T. wallichii* De C., but, for instance, the leaves are very different already at first sight; for the specific differences I might refer to Dahlstedt l.c. The exsiccatum of Bornmüller, It. persico-turcicum 1892/1893 no. 5134, type material of the species, was taken by von Handel–Mazzetti (1907) for *T. wallichii*, but his interpretation of this latter species was in a much wider sense than that of De Candolle himself.

Besides Yesd in Iran (exs. Bornmüller), Dahlstedt has mentioned localities from Turkestan. To these I can add:

Iran, Kerman: Inter Mashir 2000 m et jugum Khan-e Sorck 2580 m, K.H. et F. Rechinger, It. Iran. II, 1948 no. 3058, pro parte, Plantae P. Aellen et E. Esfandiari coll. lect.

Iran, Kerman: in limos. salsis ad Nehbid inter Kerman et Bam 2250 m, 6.5. 1948, K.H. et F. Rechinger, It. Iran. II, 1948 no. 3565; furthermore, already identified by Haglund as *T. lobulatum* in sched.: no. 3556, 3559, 3562, 3564, 3566.

Iran, Isfahan: Djulfa, in aula monast. armen. 1500 m, 20.4.1948, K.H. et F. Rechinger, It. Iran. II, 1948, no. 2662.

Beluchistan: Hooker, pro parte, and 1951 J. E. Stocks (both h. K), both identified by H.M. as *T. wallichii*.

Also material collected by W. Koelz in India (Katrain, Kulu Himalaya, 19.4. 1931, no. 1885, h.W) seems to be *T. neo-lobulatum*.

Sectio?

Taraxacum neo-kurdicum van Soest **nomen nov.;** *T. kurdicum* Haglund 1939, Botaniska Notiser: 537–538, cum icones; non von Handel–Mazzetti ex Nábělek 1925, Publ. Fac. Sc. Univ. Masaryk, Brno 52: 52.

This species only is known from Turkey!

Remark

An older name for **T. kurdicum** H.M. ex Nábělek is *T. paradoxum* H.M., 1913, Ann. Nat. Hofmus. **37**. 1.: 457, a name not being valid, as it was used earlier by Somes 1907 and Palmgren 1910.

This species is a remarkable one: a rostrum is lacking, in which respect it is comparable with 1) *T. glaciale* Huet du Pavillon (sect. *Glacialia* H.M.) and 2) *T. assemani* Boiss. and *T. primigenium* H.M. (sect. *Rhodotricha* H.M.), but there is no further direct relation to those species.

To the area of this species, hitherto Turkey, I can add:

Iraq, Erbil, K.H. Rechinger, It. Orient. 1956–1957 no. 11457.

Sectio Eu-Erythrocarpa Dahlstedt em. van Soest

In 1926 Dahlstedt has split up *Taraxaca Erythrocarpa* H.M. in two parts: sect. *Erythrosperma* and sect. *Eu-Erythrocarpa*.

The first section contained species with relatively small, reddish achenes. Afterwards Lindberg added to this group species only differing by straw-coloured achenes: Dahlstedt's section *Dissimilia*. The sect. *Erythrosperma* Dahlst. em. Lindb. f is mainly european.

The second section, *Eu-Erythrocarpa*, contains species with relatively big, reddish achenes; hitherto nine species were recognized:

T. amborum Hagl.: Greece!
T. aequilonare H.M. sens. str.: European Alps!
T. calliops Hagl.: Syria!
T. calocephalum H.M. em. Dahlst.: Greece!; also recently found in Iran: K.H. et F. Rechinger, It. Iran. II, 1948 no. 2659 and 2661. Moreover, I know plants from Greece which seem to differ only by the straw colour of the achenes.
T. duriense v.S.: Portugal!
T. hoppeanum Griseb.: Balkans!, Banat.
T. phaleratum Hagl.: Syria!, Libanon!
T. pieninicum Pawłowski: the Carpathians!
T. poliochlorum Dahlst.: Greece!

If one is prepared, likewise as in sect. *Erythrosperma*, to add to sect. *Eu-Erythrocarpa* species with non-reddish achenes, but otherwise obeying to Dahlstedt's description of this section, the following species have to be mentioned:

T. fedtschenkoi H.M.: central and western Asia! This species was classed by von Handel–Mazzetti in his section *Scariosa*.
T. protractifolium Hagl.: central Asia!
T. pseudo-nigricans H.M. sens. str.: central Asia!; in addition possibly:
T. glaucanthum (Ledeb.) De C.: Russia! to Turkestan; *T. glaucanthum* is differing from all other species of this section by a remarkable long pappus (8–9 mm).

All the species mentioned above have achenes with long and cylindrical pyramids and a well developed, rather long rostrum; the achenes, certainly in their upper half, are strongly spinulate; the pappus is white, rarely a bit dirty. Next to these already known species, I can add the following new ones: *T. aellenii* (achenes reddish), *T. pseudo-calocephalum* (reddish, rarely straw-coloured), *T. pseudo-dissimile* (straw-coloured), *T. purpurei-petiolatum* (straw-coloured), *T. spinulosum* (straw-coloured) with ssp. *calocephaloides* (reddish).

Taraxacum aellenii van Soest **spec. nov.** (Fig. 4)

Planta parva ad 6 cm alta, inferne dense longe pallide brunneo-araneosa.

Folia gramineo-viridia submaculata araneosa ad 5 cm longa petiolo violascenti inclusa, nervo mediano sordide rubescenti. Lobi laterales (utrinque 3–4) breves 4–6 mm longi triangulares saepe ± retroversi subacuti dorso undulato-denticulati, interlobiis purpureo-marginatis sublatiusculis undulato- vel crispulo-denticulatis; lobus terminalis subhastatus subobtusus interdum paulo incisus, paulo denticulatus lobulis basis retroversis subacutis ca. 3 mm longis.

Scapi floriferi foliis subaequilongi dense araneosi cuprei.

Involucrum mediocre 13 mm longum obscure viride. Squamae exteriores laxe patentes apice recurvatae ovato-lanceolatae 2–3 mm

latae conspicue sublate marginatae, pro parte callosae; squamae interiores apice purpureae obscure callosae.

Calathium subradians ad 2,5 cm diametro subpallide luteum. Ligulae marginales angustae extus stria cano-violacea notatae summis subnigrae. Antherae polliniferae; stylus et stigmata obscura. Floret vere.

Achenium rufum 4 mm longum (pyramide inclusa) superne spinulosum ceterum rugosum, in pyramiden subcylindricam 0,8 mm longam subabrupte abiens. Rostrum ca. 8 mm longum, pappus sericeo-niveus ca. 5 mm longus.

Typus: Iran, Gartenunkraut in Mahmudieh, 15 km nördlich Teheran 1250 m, IV 1948, P. Aellen (h).

T. aellenii is not very much allied to other species of this section. The dense and rather long hairy cover at the plant base is remarkable.

Taraxacum pseudo-calocephalum van Soest **spec. nov.** (Fig. 5)

Planta mediocris ca. 10 cm alta, basi fragmentis foliorum vetustorum incrassata, inferne araneoso-pilosa.

Folia canescentia subglabra lobata, petiolis nervoque mediano parte inferiore purpureo-colorato; lobi laterales (utrinque 3–4) triangulares subdentati vel denticulati acuti, interlobiis brevis saepe dentatis; lobus terminalis hastatus—subsagittatus apice subobtusus.

Scapi floriferi follis breviori basi purpurei ± araneosi.

Involucrum ± mediocre 13 mm longum viride. Squamae exteriores lanceolatae vel ovato-lanceolatae ad 2 mm latae sublate albo- vel roseolo-marginatae apice purpureae laeves vel subcallosae; squamae interiores apice sub-erosae callosae vel corniculatae.

Calathium paulo(?) radians ad 2,5 cm diametro flavum. Ligulae marginales extus stria cano-violacea notatae. Antherae polliniferae; stylus et stigmata virescenti-lutea. Floret vere-aestate.

Achenium 4 mm longum (pyramide inclusa) obscure rubrolateritium superne dense sublate spinulosum ceterum rugosum, in pyramiden cylindricam 1 mm longam (spinulis saepe praeditam) subabrupte abiens. Rostrum ca. 8 mm longum, pappus albus ca. 5–6 mm longus.

E sectione Eu-Erythrocarporum Dahlstedt.

Typus: Iran, Khorasan: Montes Kopet–Dagh. In jugo Alamli, ca. 2000 m, 3.6.1958 K.H. et F. Rechinger, It. Iran. II, 1948, Plantae P. Aellen coll. lect., no.?; also no. 4811 pro parte.

Furthermore:

Iran, Kerman: Kerman, in ruder. irrig., 1700 m, 24.4.1948, K.H. et F. Rechinger, It. Iran. II, 1948, Plantae P. Aellen et E. Esfandiari coll. lect. no. 2948, 2949, 2953.

Probably also: Iran, M. Elburs centr., in jugo Kandawan, in decl. boreal. 2700–3000 m, 26.5.1937, K.H. Rechinger, It. Iran. 1937 no. 478c pro parte; this was identified by von Handel–Mazzetti as *T. laevigatum* Willd. (h.W).

f. straminea van Soest forma nov. (achenia straminea): With the species, no. 4811, *pro parte* (see above).

This species is allied to *T. calocephalum* H.M. em. Dahlst. and still more to *T. spinulosum* ssp. *calocephaloides* m. From the first one it differs by smaller flower heads on shorter scapes, narrower and less corniculate outer bracts of the involucre; the leaves have less side lobes which are more compact together. From the second one it is distinguished by smaller, more spinulate achenes of a bit darker colour, and by narrower outer involucral bracts; also the leaf form is different.

Taraxacum pseudo-dissimile van Soest **spec. nov.** (Fig. 6); *T. subdissimile* Haglund in sched., non Dahlstedt 1933.

Planta mediocris ca. 5–10 cm alta, basi fragmentis foliorum vetustorum incrassata, inferne glabra.

Folia numerosa ad 10 cm longa (petiolo inclusa) ± glaucoviridia, juniora in costa dorsali paulo araneosa denique valde glabrescens, petiolis angustis nervoque mediano praesertim parte inferiore purpureo-colorato. Folia exteriora lingulata dentata vel sublobata, interiora lobata, lobi laterales (utrinque ca. 4) triangulares vel falcati retroversi subacuti vel peracuti mucronati integres vel dorso saepe dentati et denticulati; interlobiis ca. 0,5 cm latis; lobus terminalis saepe indeterminatus elongatus sagittato-hastatus subobtusus vel subacutus, sublobatus vel dentatus, lobuli basis retroversi acuti.

Scapi 1–4 crassi subglabri, floriferi foliis breviori.

Involucrum mediocre 10–15 mm longum ad 15 mm latum subpallide viride. Squamae exteriores laxe patentes apice recurvatae, lanceolatae apice lineares, roseolae, late albo-marginatae, laeves; squamae interiores ca. 3 mm latae membranaceo-marginatae apice obscurae laeves.

Calathium planum radians ad 3 cm diametro flavum. Ligulae marginales planae extus stria cano-violacea notatae summis obscurae. Antherae polliniferae; stylus et stigmata obscura, siccis subnigra. Floret aestate.

Achenium brunneo-stramineum 5 mm longum (pyramide inclusa) superne spinulosum ceterum laeve, in pyramiden cylindricam 2 mm longam inferne interdum spinulis praeditam sensim abiens. Rostrum ca. 7 mm longum, pappus sordide albus ca. 5 mm longus.

TYPUS: Iran, Khorasan: in collibus ad Turbat-e Haidari, 1300 m; K.H. et F. Rechinger, It. Iran. II, 1948, Plantae P. Aellen et E. Esfandiari coll. lect., no. 4328.

Furthermore:

Iran, Khorasan: Montes Hazar Masdjid 2000 m, ad fontem, 8.9.1948 K.H. et F. Rechinger, It. Iran. II, Plantae P. Aellen coll. lect., no. 5086 pro parte.

Iran, Damghan-Semnan: Zentral Elburs, im Gebiet des Kuh-i Nizwa, bei der Quelle, 10 km nördlich Djaschm, Sumpfwiese 2600 m, 28.7.1948, E. Behboudi & P. Aellen (h).

Iran, Sharud-Bustan: in declivibus australibus montium Shahvar supra Nekarman 2500 m, 20–26.6.1948; K.H. et F. Rechinger, It. Iran. II, 1948, no. 5937; the same at 2000 m, no. 5862.

Iran: Tshahar Tah, A. Gabriel (h.W); identified by H.M. as "*T. pseudonigricans*?

Iran, Kerman: Montes Djamal Bariz, inter Bam et Djiroft, ad fontem, Deh Bakri 2100 m, 8–10.5.1948; K.H. et F. Rechinger, It. Iran. II, 1948, Plantae P. Aellen et E. Esfandiari coll. lect., no. 3835 pro parte.

Iran, Mazanderan: Zentral Elburs, im Einzugsgebiet des oberen Tedschenflusses, Garten von Kom-rud bala, 60 km östlich Firuzkuh, 2400 m, E. Behboudi & P. Aellen (b); also: unterhalb Kom-rud bala, lockere Gebüschvegetation, Felsschutt.

Iraq, Erbil: Montes Quandil ad confines Persiae, ca. 36° 30′ N, 45° E., 2500 m, substr. calc., 28.7–1.8.1957; K.H. Rechinger, Itin. Orientalia 1956–1957, pro parte.

Afghanistan, Aoi Khurak, 9000′, field, 28.8.1939, W. Koelz no. 13805 (h.W).

Afghanistan, Gardiz 8000′, water course edge, 16.6.1937, W. Koelz no. 11904 (h.W).

Tadshikistan, Bapboda(?), 22.6.1945 . . . no. 422 (h. BM), pro parte.

This species, is, according to the achenes, characteristic for species of sect. *Eu-Erythrocarpa*; the achenes, however, are straw-coloured; the rather narrow outer involucral bracts are brightly washed with pink; the bracts are lacking gibbosities.

Taraxacum purpurei-petiolatum van Soest **spec. nov.** (Fig. 7)

Planta sat parva ad 8 cm alta basi subincrassata, paulo araneosa.

Folia numerosa decumbentia canescenti-viridia glabra, petiolis angustis nervoque dorsali mediano praesertim parte inferiore vinoso-colorato. Folia interiora lobata, lobi laterales (utrinque ca. 3) breves ad 5 mm longi triangulares acuti apice ± recurvatae, integres vel dorso denticulati; interlobiis subnullis; lobus terminalis elongato-sagittatus ad 3 cm longus subacutus vel acutus, retroverso-dentatus vel incisus.

Scapi ad 5, floriferi foliis breviori, glabri.

Involucrum mediocre ad 12 mm longum ad 15 mm latum crassiusculum viride. Squamae exteriores adpressae ovatae 4 mm longae anguste albo-marginatae, apice purpureae laeves; squamae interiores late lineares membranaceo-marginatae pro parte callosae.

Calathium planum radians ca. 2 cm diametro flavum. Ligulae marginales extus stria cano-violacea notatae. Antherae polliniferae; stylus et stigmata obscura, siccis subnigra. Floret aestate.

Achenium stramineum 4 mm longum (pyramide inclusa) superne subgrosse spinulosum ceterum rugosum—basi laeve, in pyramiden cylindricam 1 mm longam subabrupte abiens. Rostrum ca. 7 mm longum, pappus albus ca. 5 mm longus.

Typus: Iran, Khorasan: Montes Hazar Masdjid, 8–9.6.1948; K.H. et F. Rechinger, It. Iran. II, 1948, Plantae P. Aellen coll. lect., no. 5086, pro parte.

Furthermore:

Iran, Inter Kerman et Saidabad (Sirdjan) inter Mashiz 2000 m et jugum Khan-e Sorck 2580 m, 27.4.1948; K.H. et F. Rechinger It. Iran. II, 1948, Plantae P. Aellen et E. Esfandiari coll. lect., no. 3058 pro parte.

The achenes of this species are smaller in size than is normal in this section and therefore there might be some reason to place it in sect. *Erythrosperma* (*Dissimila*), but in the other characters it seems

more related to the *Eu-Erythrocarpa*; it reminds a bit of *T. pseudo-dissimile*, but it is smaller in practically all its parts, the achenes are more spinulate, the involucral bracts are different in shape and colour, the outer ones appressed.

Taraxacum spinulosum van Soest **spec. nov,** (Fig. 8)

Planta mediocris ca. 10 cm alta subglabra.

Folia numerosa canescenti-viridia subscariosa lobata, petiolis subangustis nervoque mediano praesertim parte inferiore purpureo-colorato. Lobi laterales (utrinque ca. 4) approximatis, hamati vel triangulares ad 1,5 cm longi utrinque valde argute dentati et denticulati subspinulosi apice mucronati; lobus terminalis hastatus interdum grosse dentatus ad 1,5 cm longus, lobulo apicali subacuto mucronato, lobulis basalibus acutis mucronatis.

Scapi ad 4 subcrassi, floriferi foliis subaequilongi, sub involucro araneoso-pilosi.

Involucrum mediocre ca. 13 mm longum ca. 15 mm latum basi rotundatum. Squamae exteriores erecto-patentes apice recurvatae ovato-lanceolatae 7 mm longae ad 3 mm latae inconspicue sublate marginatae subcallosae, apice purpureae; squamae interiores apice violaceae callosae vel corniculatae.

Calathium paulo radians ca. 2,5 cm diametro, flavum. Ligulae marginales planae extus stria cano-violacea notatae. Antherae polliniferae, stylus et stigmata fusco-virescentia denique subobscura. Floret aestate.

Achenium submagnum 4 mm (pyramide exclusa) dilute brunneum, superne grosse late squamulis saepe tricuspidatis praeditum, ceterum costulatum, rugosum—basi laeve, in pyramiden cylindricam 1,5 mm longam (spinulis saepe praeditam) abrupte abiens. Rostrum ca. 10 mm longum; pappus albus, 6–7 mm longus.

Typus: Iran, Montes Elburs centr.: in jugo Kandawan, in declivibus borealibus, 2400 m, 9.6.1937; K.H. Rechinger fil., It. Iran. 1937 no. 957 h; also 957 a.

Furthermore:

Iran, Sharud-Bustam: in declivibus australibus montium Shahvar ad Nekarman, 2000 m, 20–26.7.1948; K.H. et F. Rechinger, It. Iran. II, 1948 no. 5827.

Iran, Khorasan: in jugo Alamli 2000 m, 3.6.1948; K.H. et F. Rechinger, It. Iran. II, 1948, Plantae P. Aellen coll. lect., no. 4807.

Iran, Khorasan: Montes Hazar Masdjid, 8–9.6.1948; K.H. et F. Rechinger, It. Iran. II, 1948, Pl. P. Aellen coll. lect., pro parte.

This species is allied to *T. pseudo-nigricans* H.M. and *T. pseudo-dissimile* m; from both it is easily distinguished by the mucronate, nearly spinulate leaves (with clear purple petioles); those of *T. pseudo-nigricans* have palish petioles, its involucra have darker greyish-green outer bracts, with stronger cornicula, its achenes have thinner, more acute spinula's.

The petioles of the leaves in *T. pseudo-dissimile* also are purplish; the outer involucral bracts, pale and purplish to pink-coloured as in

T. spinulosum but much more outspoken, are elongated in a narrow top; its achenes are more sharply spinulate, its rostrum is shorter.

T. spinulosum is found also with darker brown achenes:

Iran, Montes Elburs centr.: in jugo Kandawan 2700–3000 m, 26.5.1937; K.H. Rechniger fil., Iter Iran. 1937 no. 478 b; in h. W, von Handel–Mazzetti identified this wrongly as *T. officinale*.

Furthermore this species is known with brown-red achenes; the collected plants show a great similarity to the others, only the form of the achenes also is slightly different:

ssp. **calocephaloides** van Soest **nov. subspec.**

Differt ab *T. spinuloso* typico: acheniis badio-rubris, superne argute spinulosis, in pyramiden subsensim abiens; rostro 12 mm longo, pappo sordide albo.

Typus: Iran, Montes Elburs centr., in jugo Kandawan, in declivibus borealibus 2400 m, 9.6.1937; K.H. Rechniger fil., Iter Iran., 1937, no. 957 c.

Furthermore:

Iran, Mazanderan: Elburs, feuchter Graben bei Gaduk 2200 m, 20.7.1948, E. Behboudi & P. Aellen (h).

This subspecies is a bit similar to *T. pseudo-calocephalum* m., but not only distinct by the leaf characteristics (mucronate to nearly spinulate, f.i.), but moreover by its bigger and darker achenes.

Sectio Erythrosperma Dahlst. em. Lindb. f.

Taraxacum persicum van Soest **spec. nov.** (Fig. 9)

Planta parva 6–10 cm alta basi ± araneosa.

Folia ad 5 cm longa (petiolis pallidis inclusa) griseo-viridia subglabra lobata; lobi laterales (utrinque ca. 3) breve triangulares ± retroversi acuti dorso breve dentato vel denticulato vel integro; interlobiis subnullis interdum dentatis; lobus terminalis indeterminatus vel sublobatus ad 12 mm longus obtusus paulo dentatus vel denticulatus, lobuli basis ± acuti retroversi.

Scapi 1–2 paulo araneosi.

Involucrum obscure viride 11–12 mm longum 7–8 mm latum. Squamae pro parte subcallosae, exteriores laxe adpressae—reflexopatentes, lanceolatae ca. 4 mm longae inconspicue anguste marginatae; squamae interiores late lineares membranaceo-marginatae laeves.

Calathium paulo radians pallide luteum. Ligulae marginales planae, extus stria atroviolacea notatae. Antherae ± polliniferae; stylus et stigmata subobscura. Floret vere.

Achenium parvum 3,5 mm longum (pyramide inclusa) rubrolateritium superne spinulosum, in pyramiden cylindricam 0,6 mm longam subabrupte abiens. Rostrum 8–9 mm longum, pappus albus 5 mm longus.

E sectione Erythrospermorum Dahlstedt em. Lindberg f.

Typus: Iran, Asterabad: Bender Ges, in pascuis, 7 & 31.3.1901,

P. Sintenis, It. Transcaspico-persicum 1900/1901, no. 1433 (h.L, h.B.M. and h.K).

Furthermore:

Syria, inter urbum Haleb (Aleppo) et vicum Meskene ad Euphratem, ad rivum prope Adschuk, 400 m, 25.3.1010 von Handel-Mazzetti, Mesopotamien-Exped. 341, pro *T. laevigato*.

This species is related to *T. silesiacum* Dahlst.; the leaves are more or less similar. The outer involucral bracts have only a very narrow margine, the bracts are practically without gibbosities; pollen is present and the rostrum is slightly longer. *T. silesiacum* is known from practically the whole of Europe, except the mediterranean region; furthermore it is introduced in the northeastern part of United States of America.

Sectio Spuria De C.
Taraxacum koelzü van Soest **spec. nov.** (Fig. 10)

Planta ca. 10 cm alta, basi dense araneosa.

Folia decumbentia subcanescentia denique parce araneosa, subcoriacea, in petiolis (nervoque mediano parte inferiore purpureo-colorato) longe truncata. Folia exteriora lingulata subintegra, interiora lobata; lobi laterales (utrinque ca. 3) anguste deltoidei vel lingulati acuti integres; interlobiis ad 7 mm longis ad 5 mm latis minute dentatis vel denticulatis; lobus terminalis $\pm$ tripartitus, lobuli basis $\pm$ deltoidei acuti, lobulo apicali subelongato subacuto integro.

Scapi ca. 2, dense araneosi denique recurvati.

Involucrum ca. 15 mm longum 12 mm latum. Squamae exteriores laxe adpressae numerosae imbricatae ovatae 3–7 mm longae sordide roseolo-coloratae late albo-marginatae apice purpureae, interdum inconspicue callosae; squamae interiores late lineares, late luteoviride—membranaceo-marginatae apice purpureo-marginatae laeves.

Calathium paulo (?) radians 2,5 cm diametro flavum. Ligulae marginales planae 2 cm longae extus stria saturate purpurea ornatae. Antherae polliniferae; stylus et stigmata aurea. Floret autumno.

Achenium stramineum ca. 7 mm longum (pyramide inclusa) superne breve spinulosum, in pyramiden conico-cylindricam longam, spinulis praeditam sensim abiens. Rostrum ca. 7 mm longum, pappus sericeus ca. 9 mm longus.

E sectione Spuriorum De Candolle.

Typus: Afghanistan: Burchao Pass, dry slope 10.000′, 13.10. 1939, W. Koelz 14142 (h.W).

In section *Spuria T. koelzii* is allied to *T. farinosum* Haussknecht & Bornmüller and to *T. roseum* Bornmüller ex H.M. The first one has small achenes, is flowering in spring and has halophytic and xerophytic characters. The second one is easily distinguished by its clear pink flowers. The three other species of this section are taller in size and bigger in dimensions: *T. syriacum* Boissier, *T. montanum* (C.A. Mey.) H.M. and *T. neospurium* m, all flowering in summer time.

Taraxacum neo-spurium van Soest **nov. spec.** (Fig. 11)

Planta ad 8 cm alta araneosa.

Folia numerosa decumbentia subobscure viridia lobata; lobi laterales ad 1,5 cm longi subfalcati acuti dorso dentati; interlobiis ad 1 cm longis ad 3 mm latis saepe crispulis, denticulatis vel interdum dentatis; lobus terminalis deltoideo-subsagittatus, lobuli basis paulo retroversi acuti integres vel 1 dente muniti, lobulo apicali acuto integro.

Scapi ad 6 interdum furcati, dense araneosi foliis breviori denique recurvati.

Involucrum magnum ad 2,5 cm longum ad 1,5 cm latum sordide viride. Squamae exteriores laxe adpressae imbricatae ovato-lanceolatae acutae ad 1 cm longae late pallide viride-marginatae sub apice purpureo-callosae; squamae interiores late lineares late membranaceo-marginatae, apice rufo-purpureae.

Calathium paulo radians flavum (?). Ligulae marginales angustae, extus concolores vel stria ± purpurea notatae. Antherae vacuae; stylus et stigmata subpurpurea. Floret augusto.

Achenium fulvum ca. 9 mm longum (pyramide inclusa) superne breve et late spinulosum, in pyramiden conicam longam sensim abiens. Rostrum ca. 9 mm longum; pappus sordide albus ca. 10 mm longus.

E sectione Spuriorum De Candolle.

Typus: Iraq, Kurdistan: distr. Erbil, Mons Helgurd ad confines Persiae ca 36° 40′ N, 44° 50′ E. in valle supra pagum Nowanda, 2600–3000 m; 10–14.8.1957, K.H. Rechinger It. Orientalia 1956/57 no. 11891.

According to the involucrum, the achenes and the dense indument this species is characteristic for the section *Spuria*; the leaves are more likely to those of section *Vulgaria* Dahlst. The colour of the achenes is exceptional in *Spuria*. The purple colour of the stigma's is very rare in the whole genus; I only know the same colour in *T. fontaniforme* m. in section *Cucullata* m from the European Alps, a species that has no relation to *T. neo-spurium*. The sometimes furcate scapes appear also rather normally in *T. montanum* (C.A.M.) De C.; in other sections furcation only represents a monstruous development.

SUMMARY

Eleven species of *Taraxacum* and one subspecies are described. For nomenclatoral reason two species have changed in name.

Sect.?; *T. iranicum* v.S.

Sect.?: *T. rechingeri* v.S.

Sect. *Macrocornuta* v.S. nov. sect. (typus: *T. wallichii* De C.), including *T. monochlamydeum* H.M. em Hagl., *T. neo-lobulatum* v.S. nov. nom. (*T. lobulatum* Dahlst., non Brenner) and new: *T. afghanicum* v.S.

Sect.?: *T. neo-kurdicum* v.S. nov. nom. (*T. kurdicum* Hagl., non H.M. ex Nábělek).

Sect. *Eu-Erythrocarpa* Dahlst. em. v.S.: a number of species with non-reddish achenes are joined to this group: *T. fedtschenkoi* H.M., *T. protractifolium* Hagl., *T. pseudo-nigricans* H.M. sens. strict.; new species are added: *T. aellenii* v.S., *T.*

pseudo-calocephalum v.S., *T. pseudo-dissimile* v.S., *T. purpurei-petiolatum* v.S., *T. spinulosum* v.S. with ssp. *calocephaloides* v.S.

Sect. *Erythrosperma* Dahlst. em. Lindb. f.: *T. persicum* v.S.

Sect. *Spuria* De C.: *T. koelzii* v.S., *T. neo-spurium* v.S.

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The author expresses his gratitude to Mr. P. Aellen and Dr. K. H. Rechinger for sending important material to him in loan, to Dr. S. Ahlner (h. Stockholm) for bringing to his disposal type material of some species, described by Haglund. He is also thankful to Mr. C. L. Marks for preparing the photographs (published here by courtesy of the Director of the Rijksherbarium, Leiden).

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Fig. 1. Taraxacum iranicum v.S.; type material.

Fig. 2. Taraxacum rechingeri v.S.; type material.

Fig. 3. Taraxacum afghanicum v.S.; type material.

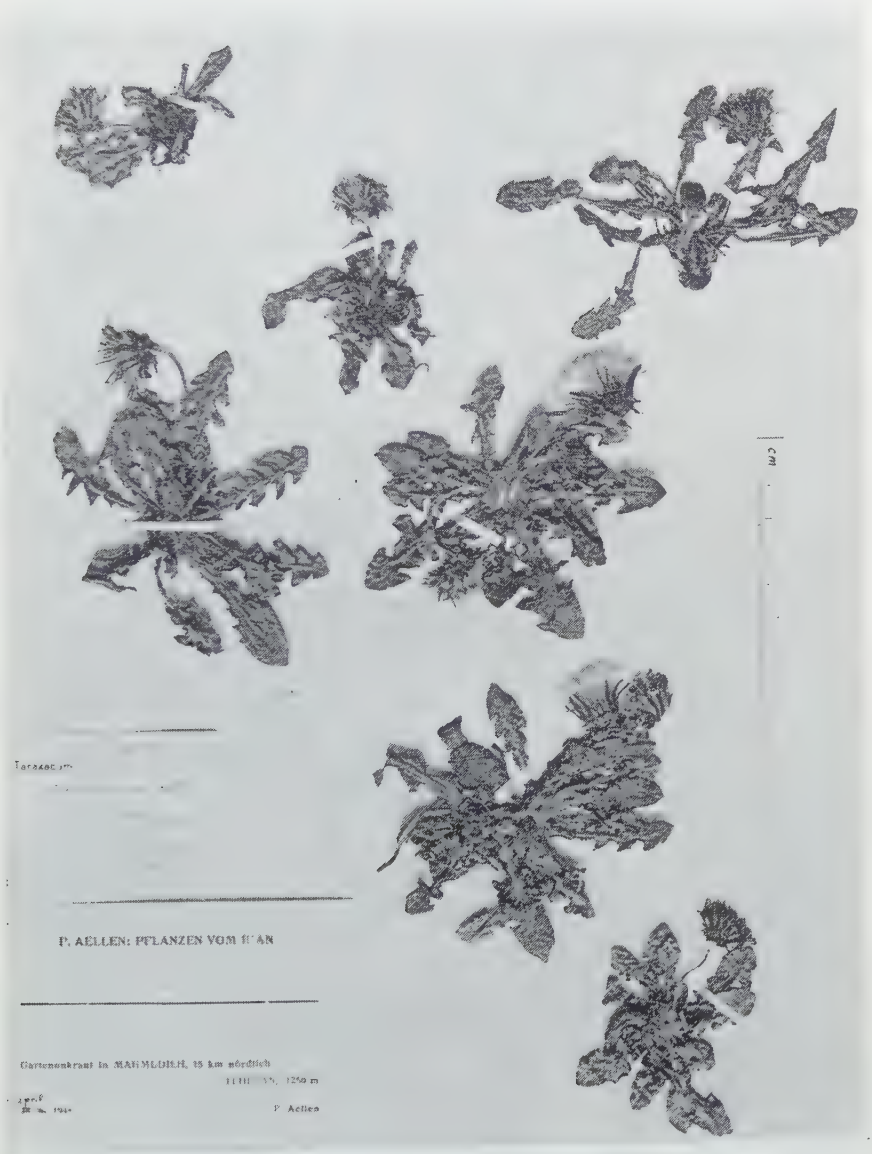

Fig. 4. Taraxacum aellenii v.S.; type material.

Fig. 5. Taraxacum pseudo-calocephalum v.S.; type material.

Fig. 6. Taraxacum pseudo-dissimile v.S.; type material.

Fig. 7. Taraxacum purpurei-petiolatum v.S.; type material.

Fig. 8. Taraxacum spinulosum v.S.; type material.

Fig. 9. Taraxacum persicum v.S.; type material.

Fig. 10. Taraxacum koelzii v.S.; type material.

Fig. 11. Taraxacum neo-spurium v.S.; type material.

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CHROMOSOME NUMBERS IN LEGUMINOUS PLANTS

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During the last few years a number of chromosome counts have been made in the family of the *Leguminosae*, some of which are not yet reported in literature, whereas others deviate from what has been stated earlier.

Taking into account that a more exhaustive investigation cannot be tackled with in the near future, or is impossible owing to the lack of more material from the genus, there seems to be a justification to publish these counts at present without further comment.

In a few cases (*Abrus*) a revision of the related herbarium material enabled to re-establish the nomenclature.

The numbers added refer to the herbarium specimens collected at the finding-place or reared afterwards from seed in the hothouses at the Laboratory of Tropical Agriculture of the State Agricultural University, Wageningen.

When discrepancies from earlier statements were noticed, the author(s) and the year of publication have been indicated: particulars on these publications may be found in the two editions of Darlington's Chromosome Atlas (1945, 1955) and in the Index to Plant Chromosome Numbers 1956–1958 published by the California Botanical Society.

	Herbarium No.	Origin	Year of collection	2n	
Mimosaceae					
1 *Acacia juniperina* DC.		Hortus Botanicus, Amsterdam	1955	26	
2 ,, *verticillata* Willd.		Hortus Botanicus, Amsterdam	1955	28	26 Tjio 1948
3 *Leucaena glabrata* Rose	Wonder-ley 17	Mexico, Herbarium Utrecht	1941	± 104	
Caesalpiniaceae					
4 *Cassia absus* L.	54011	Adiopodoumé, Ivory Coast	1954	28	
5 ,, *acutifolia* Delile		Bot. Garden, Poznan	1956	26	28 Milovidov & Štochová 1958
6 ,, *angustifolia* Vahl.		Madaus–Köln	1956	28	28 Sampath & Ramanathan 1949; n = 13 Mehra & Sobti 1955
7 ,, *obtusifolia* L.	54023	Adiopodoumé, Ivory Coast	1954	24	26 Pantulu 1942
8 ,, *occidentalis* L.	54030	Adiopodoumé, Ivory Coast	1954	26	26 Muto 1929; 28 Pantulu 1940; n = 14 Turner 1956
9 ,, *podocarpa* G. & P.	54066	Brafouédi, Ivory Coast	1954	28	
10 ,, *rotundifolia* Pers.	57033	Shika (from Plateau) Nrth. Nigeria	1957	14	
Papilionaceae					
Galegeae					
11 *Notospartium glabrescens* Petrie		Hortus Botanicus, Amsterdam	1955	28	
Vicieae					
12 *Abrus canescens* Welw. ex Baker	54074	Oroumbo Boka, Ivory Coast	1954	22	
13 do.	57095	Boukoko, Oubangui	1957	22	
14 *Abrus fruticulosus* Wall. ex W. & A. var. *villosulus* (Miq.) Breteler	54061	Adiopodoumé, Ivory Coast	1954	22	
15 do.	57138	Abba, Dahomey	1957	22	

Trifolieae						
16	*Trigonella arabica* Del.		Israël	1953	16	
Phaseoleae						
17	*Amphicarpa comosa* Don.		Bot. Garten, Zürich	1956	22	
18	*Canavalia obtusifolia* DC.	54053	Azuretti, Ivory Coast	1954	22	
19	*Clitoria rubiginosa* Juss. ex Pers.	57169	Moossou, Ivory Coast	1957	24	
20	*Galactia tenuiflora* Wight & Arnold	57127	Pobé, Dahomey	1957	22	
21	*Psophocarpus palmettorum* G. & P.	57113	Boukoko, Oubangui	1957	20	
22	,, *palustris* Desv.		Medan, Sumatra	1957	20	
23	*Teramnus uncinatus* Swartz	57068	Shika, Nrth. Nigeria from Kitale, Kenya	1957	28	
Hedysareae						
24	*Ormocarpum guineense* Hutch. & Dalz.	57171	Moossou, Ivory Coast	1957	26	
25	*Uraria picta* Desv.	57106	Boukoko, Oubangui	1957	22	
Genisteae						
25	*Crotalaria calycina* Schrank.	57176	Dabou, Ivory Coast	1957	16	
26	,, *cylindrocarpa* Walp.	57072	Samaru, Nrth. Nigeria	1957	16	
27	,, *ebenoides* Walp.	57003	Jos, Nrth. Nigeria	1957	16	
28	,, *glauca* Willd.	57036	Shika, Nrth. Nigeria (from Plateau)	1957	14–16	
29	do.	57076	Samaru, Nrth. Nigeria	1957	16	
30	*Crotalaria goreensis* G. & P.	57038	Shika, Nrth. Nigeria (from Plateau)	1957	16	
31	do.	57073	Samaru, Nrth. Nigeria	1957	16	
32	do.	57088	Kano, Nrth. Nigeria	1957	16	
33	*Crotalaria lachnocarpa* Hochst.	57040	Shika, Nrth. Nigeria (from Plateau)	1957	16	
34	,, "*mejusei*" (= Muyssi Hort. aff. *C. usuramoensis* Bak. f.)	57167	Adiopodoumé (from Bouaké) Ivory Coast	1957	16	
35	,, *naragutensis* Hutch.	57068	Kano, Nrth. Nigeria	1957	16	
36	,, *ochroleuca* G. Don.	57070	Samaru, Nrth. Nigeria	1957	16	
37	,, *Perrotettii* DC.	57004	Jos, Nrth. Nigeria	1957	16	
38	,, *retusa* L.	57050	Shika, Nrth. Nigeria (from U.S.A.)	1957	16	
39	do.	57079	Samaru, Nrth. Nigeria	1957	16	
40	do.	57165	Adiopodoumé, Ivory Coast	1957	16	
41	*Crotalaria senegalensis* Bacle ex DC.	57051	Shika, Nrth. Nigeria	1957	16	
42	,, *spartea* Planch.	57103	Boukoko, Oubangui	1957	16	
43	,, *Vogelii* Benth.	57173	Dabou, Ivory Coast	1957	16	
44	*Genista floribunda* Hort.		Antibes	1953	± 48	
45	*Ulex gallii* Planch.		Antibes	1953	96	80 De Castro 1943

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THE SEED OF CARDIOSPERMUM HALICACABUM L. A CRITICISM

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(*received August 9th, 1959*)

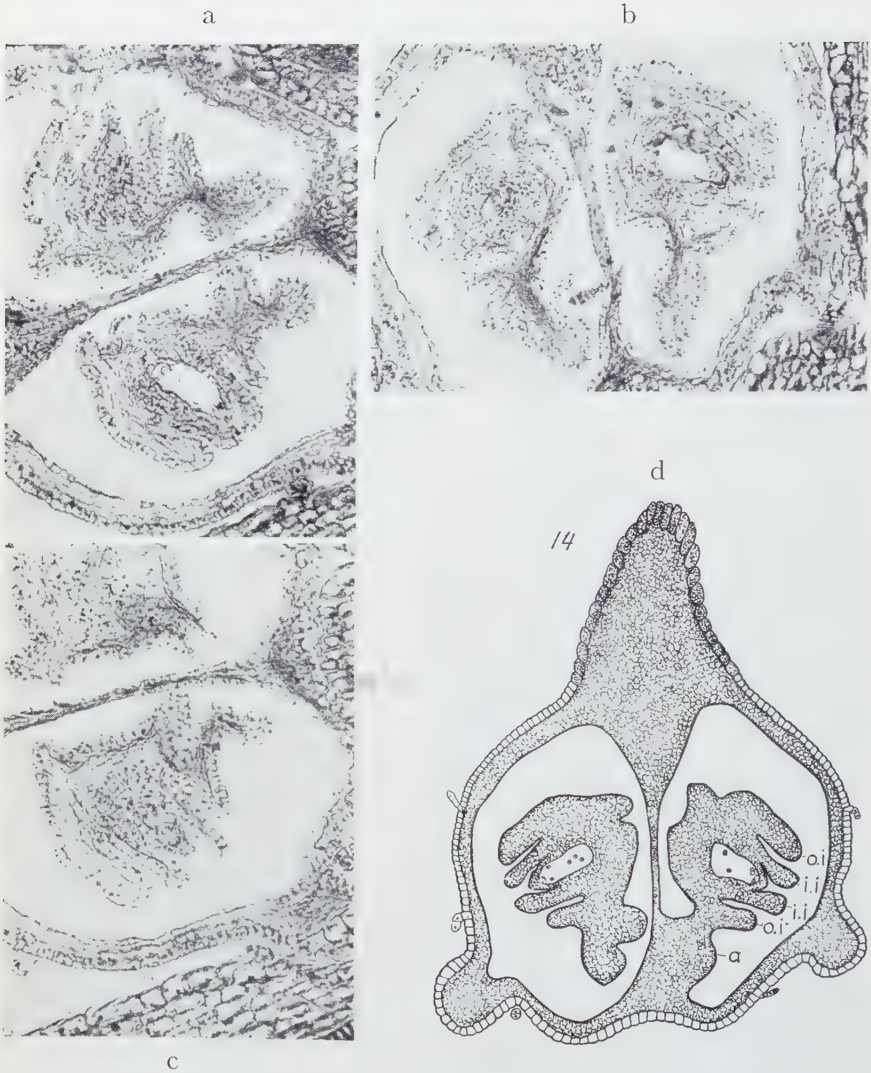
Van der Pijl (1957) has criticized my paper (1946) on the "Embryology of Cardiospermum halicacabum L." in different points. I feel it is my duty to discuss the ideas which van der Pijl put forward with regard to my figures, which were drawn with the aid of a camera lucida. I wish to make three remarks in this respect.

1. Van der Pijl says on page 625 that "Kadry interpreted his Fig. 15*a* as showing a micropylar beak, a massive mucilaginous tissue originating from the tip of the nucellus and from part of the inner integument. Kadry contested the correctness of Guérin's opinion that this beak surrounds a micropylar canal. Guerin's Fig. 1, the structure generally found in the ovules of the family and my own sections through these ovules make it clear that this beak is nothing but the tip of the solid looking inner integument, and that the nucellus has been resorbed by this time (Fig. 6*b*)". I have expounded my view on page 116 of my paper (1946) where it was stated that "After the organisation of the embryosac, the cells at the tip of the nucellus and some of the cells of the inner integument which encircle the micropyle, form a mucilaginous mass through which the pollen tube enters. In consequence of that, the micropylar canal becomes entirely closed. This latter view is in contrast to Guérin's observation (1901). The author made continuous series of longitudinal and transverse sections of different stages of advanced ovules passing through the region of the micropyle (Fig. 15). A careful examination of these sections leaves no doubt that the pollen tube passes through a continuous micropylar tissue". Van der Pijl objects to my interpretation. Very recently I have prepared photomicrographs which undoubtedly show the incorrectness of van der Pijl's views.

Photomicrograph 1 shows mature ovules in the period of fertilization; the massive tissue of the nucellus, which has undergone a fusion with the cells of the inner integument, forms a mucilaginous tissue. Neither in these sections nor in the rest of the series obtained from the same ovules, any trace of a micropylar canal was observed. The same result was obtained with numerous other ovules that were studied during and after fertilization. Photomicrograph (1*a*) was taken from a section passing through the axis of the ovule, and here, therefore, the micropylar canal ought to be visible if it were present as van der Pijl suggested.

2. Van der Pijl says (on page 625); "At this place inside the curvature the outer integument, though recognizable in young ovules,

Photomicrograph. I. *Cardiospermum halicacabum.* a-d. Longitudinal sections through three different ovules made at the time of fertilization and photographed at different magnifications, showing the massive nucellus fused with the cells of the inner integument and forming with the latter the mucilaginous tissue, and the absence of a micropylar canal, the inner part of the outer integument appears to be indistinguishable, a. showing the axial part of the ovule in which the micropylar canal should be visible if it were present, b. showing a pollen tube with its two male nuclei, c. a higher magnification of b. The latter corresponds to Fig. 15*a* of my paper (1946) and is reproduced to prove its correctness.



Photomicrograph 2. *Cardiospermum halicacabum.* a-c. A series of longitudinal sections through a young ovary, showing two ovules each with the inner as well as the outer integument fully developed, d. photomicrograph corresponding to Fig. 14 of my paper (1946). It undoubtedly shows that the interpretation of van der Pijl is wrong.

is said to become indistinguishable in older ovules. We can, however, discern it in Kadry's drawing and it is clearly recognizable in Guérin's drawings as well as in my own sections (Fig. 6)". I have described in detail the development of the outer integument during the different stages of the ovule. I said (on page 114), "During the formation of the four-nucleate stage of the embryo-sac, the two integuments become more visible on both sides, and the ovule represents an intermediate stage of the anatropous type (Figs. 8 and 14). At this stage, the lower part of the outer integument on the side towards the funiculus become very distinct. This latter part of the outer integument was already seen in all examined cases of the same stages, though COULTER and CHAMBERLAIN (1912) stated (on page 57) that, in anatropous ovules with two integuments, the outer one is not developed on the side toward the funiculus." I have distinguished the inner part of the outer integument of the young ovules very clearly and it is clearly shown in my drawings. But with regard to the older ovules, I said (on page 116), "During the curvature of the ovule the inner part of the outer integument fuses with the adjacent tissue and cannot be easily distinguished on this side, while its outer part is fully developed and is more visible on the free side of the ovule". This means that in the older ovules the inner part of the outer integument becomes indistinguishable, and it is therefore not shown in my drawings of the older ovules.

Photomicrograph (1*c*) shows a longitudinal section of an anatropous ovule which strongly support this view. Van der Pijl's section may have been cut in some distance from the axis.

3. Van der Pijl states (on page 626) that, "There is no thin, true funicle as figured in Kadry's Fig. 14. The figures in Payer's Organogenie (1857) already proved this". Although I have not discussed the funicle in my paper (1946), yet I may point out that we can not overlook the fact that Fig. 14 is drawn from a true section, and we cannot discard any feature shown in it, even if it contradicts the findings of other authors. This figure was drawn with the aid of a camera lucida (the same as all the drawings in my paper (1946). In corroboration of this Fig 14 which van der Pijl doubts, I reproduce a photomicrograph of the section through this ovary (2). Both the camera lucida drawings and the photomicrographs are absolutely identical, and support completely the conclusions I have drawn before.

CONCLUSION

The present criticism aims at removing the objections raised by van der Pijl. The accompanying photomicrographs give reliable evidence in support of the following conclusions.

1. In the mature ovule there is no micropylar canal, its place being occupied by nucellar cells and cells of the inner integument which together form the mucilaginous tissue.
2. In the older ovules the inner part of the outer integument becomes indistinguishable.
3. Fig. 14 of my paper (1946) is an exact reproduction of a section.

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COMMENT ON THE FOREGOING PAPER BY A. R. KADRY

L. VAN DER PIJL

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Although the criticism of Kadry remains confined to some minor points and does not affect the main issue of my study, it seems desirable to consider his objections briefly.

Ad 1. A very narrow micropylar canal (cf. *Harpullia*), especially when it runs obliquely to the axis, is apt to escape the attention in a longitudinal section. A negative finding in a section as shown in Kadry's photograph 1*b*, does not prove much, as the section may easily have missed the canal; it can not be used to disprove a positive finding by another author (Guérin). For the rest I do not understand why my "views should be corrected". I did not object to any interpretation, only ventured to place Guérin's opinion beside that of Kadry, and I myself spoke neutrally of a solid-looking integument, the point being of no interest for the matter under discussion.

The photograph certainly does not prove (as said in the caption) that the "beak" is a fusion between nucellus and integument. This statement consists of a mixture of observation and interpretation (just as his statement in the contested main issue on the origin of the "aril"). The conclusion of Guérin, based on ontogenetical investigation which proves the resorption of the nucellus, can not be dismissed by this photograph. I may add that I found the same resorption in the ontogeny of the ovule in other Sapindaceae.

Ad 2. Photograph 1*c* shows only the tip of the beak. For this region I too mentioned the absence of a funicular outer integument, so that the photograph seems unneccessary. The funicular outer integument was described by me as present nearer to the base, inside the curve, whereas Kadry now says that in older ovules the whole inner part of the outer integument becomes indistinguishable. However, in Kadry's microphotograph 1*b* this part is clearly distinguishable.

Ad 3. Photograph 2*b* does not agree with the older Fig. 14 of Kadry, to which it refers, and with which it is said to be absolutely identical. In Fig. 14 the place of origin of the aril (my main point) seems to be reconstructed. Fig. 14 seems to be a combination of the photographs 2*a* and 2*b*. It can not have been (as said in the text) "drawn from a true section" as it is impossible that in a large trilocular young fruit two seeds are cut medially in one section.

I fail to see how the photographs could possibly disprove my thesis that the basal folds on which the ovules are inserted, are the "Querzones" of peltate carpels, and that there are no true funicles. In the right half of Fig. 14 (more or less corresponding to photograph 2*b*) a funicle is clearly absent.

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ANATOMICAL CHANGES IN CLADODES OF PHYLLOCACTUS HYBR. IN RELATION TO FLOWERING

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Introduction

Phyllocactus plants coming into flower exhibit thickened strands, often coloured a purple tone by anthocyanins, which run from the "midrib" of the leaf-like stem (cladode) to the budding areoles (Plate 1*a*). During the progress of flower-bud growth the strands increase in thickness. Resting areoles do not show this phenomenon. It was first described by Vöchting (1873–'74) for species of *Rhipsalis* and related genera and by Schumann (1895) for *Phyllocactus*. Anatomical data, however, are, as far as the present authors are aware, not available. The purpose of the present paper is to give some information as to the anatomical background of the observed phenomenon.

Material and methods

The plant used in our investigation is grown in large quantities in the Netherlands flower centre of Aalsmeer, under the name of *Phyllocactus ackermannii* (Haw.) Salm–Dyck. This is certainly not the correct name, since the plant in question is not a true species but a highly hybridized cultivar. The colour of the plant is bright green. The basal part of the leaf-like stems (cladodes) is cylindrical. The flowers measure about five inches in diameter and are of a bright orange-toned red colour. Plants well-set with flower buds in various stages of development were obtained from the local market and kept in the greenhouses of the Botanical Garden.

To obtain information on the gross anatomy of the shoots after decolouration and dehydration in a series of concentrations of aethanol, they were made transparent by means of methylbenzoate. In preparing slides, great care had to be taken with the fixation and the embedding of the material. As a matter of fact, this type of material may cause difficulties in connection with the presence of huge amounts of mucilage and with the marked differences in firmness of the cell walls of the tissues involved.

Fragments of stems with resting areoles and with areoles bearing flowerbuds in different stages of development, from very small buds to fully expanded flowers, were fixed for 48 hours in vacuo in a mixture of 12.5 cc glacial acetic acid, 12.5 cc propionic acid, 25 cc formaline and 450 cc distilled water. After fixation the material was washed for 4 hours in running tap water, after which the fragments were rinsed with distilled water and dehydrated according to Johansen's

method (1940) with tertiary butanol. Impregnation with paraffin wax was carried out very carefully. Small pieces of aerated paraffin wax (melting point 50° C) were added hourly to the tertiary butanol until saturation was reached. After evaporation of the butanol the paraffin wax was replaced first by one with a melting point of 54° C and later by one with a melting point of 64° C. After renewing this last wax once or twice, embedding proceeded in paraffin wax of the same melting point.

Cut into 10 μ thick sections, the material was deprived of paraffin in a range of xylene-tertiary butanol mixtures. Via 70 % and 50 % aethanol the sections were placed in a 1 % saffranin solution in 50 % aethanol in which they remained for 16 hours. Washed successively in 50, 70 and 95 % aethanol, the sections were counterstained for 1 minute in a 1 % solution of fast green in 96 % aethanol. Via a tertiary butanol-xylene range, the sections were finally enclosed in Caedax. Notwithstanding careful treatment, deformation of parenchymatous tissue as well as some rupture of the very tender-walled cambial zone was unavoidable.

Morphology and gross anatomy

The stems of *Phyllocactus* species have a more or less leaf-like character. They are green in colour, are flattened, and show a distinct "mid-rib" with "lateral veins" interconnected by many very thin secondary veins, resulting in a sort of network. Such stems have been termed cladodes. The "midrib" is the central cylinder of the stem, while the major "lateral veins" are formed by leaf and branch traces running through the cortex of the stem towards the very small scale-like rudimentary leaves, the so-called areoles being the axillary buds belonging to these leaves. From such areoles flower-buds or new stems may develop. Moreover, minor "lateral veins" originate from the central cylinder. Together with the leaf traces, the minor lateral veins and the secondary veins form the cortical vascular system.

By using the clearing method described in the preceding section, the whole vascular system can be demonstrated. It appears that leaf and branch traces anastomose to a varying degree. Moreover, it can be seen that the development of flower-buds and of the correponding vascular system is simultaneous (Plate 2).

Anatomy

General survey

The major object of the present publication is to describe the changes which take place in branch traces in relation to the development of flowers. Since the changes observed are the direct cause of certain alterations in the cortical region, a short description of the anatomy of the whole shoot will be given.

Under the epidermis, which is provided with a well-developed cuticle the peripheral layer of the cortex consists of small cells forming a distinct hypodermis (Plate 1*b*). The remaining part of the well-

developed cortex which gives the plant its succulent character is of a parenchymatous nature. A special feature is the presence of numerous mucilage idioblasts which have already drawn the attention of early plant anatomists (*see* Stewart, 1919). This type of cell is characterized by large dimensions and the mucilaginous substance which it contains.

Embedded in the cortical tissue are the branch and leaf traces and the minor veins. These are composed of collateral vascular bundles, whose number varies with the thickness of the various strands. It is striking that during the development of flower-buds from resting areoles not only the branch traces but also the leaf traces and their anastomoses gain in importance (Plate 2).

Vascularization of the resting areole

Close to its insertion, the branch trace consists of three small vascular bundles separated by parenchyma. The number of bundles increases considerably towards the areole. This is due in part to division of the original three bundles and in part to the contribution to the configuration of anastomoses from the leaf traces. Finally, very close to the areole minor bundles from the cortical system may enter the ring of vascular bundles. The amount of vascular tissue increases towards the areole not only by the increase of the number of vascular bundles but also, because of the occurence of some secondary growth (Plate 4*a*). As a result, localized dilatation of cortical tissue takes place. Details will be given in a separate paragraph.

Vascularization of the flowering areole

The most conspicuous phenomenon is the very extensive secondary growth that occurs during the period between initiation of flower-buds and anthesis (Plate 2). This growth takes place over the whole distance between insertion site of the traces and the areole, with maximum development close to the areole (Plate 4*b*). Secondary phloem and xylem are formed in considerable quantities by the action of fascicular cambium. True interfascicular cambium is not formed. The cells of the interfascicular regions divide several times, giving rise to irregular radial rows of cells which clearly show their origin (Plate 5*a*). Although it is not absolutely absent in traces leading to resting areoles, those which end in flowering areoles exhibit the following feature: corresponding to and localized at the periphery of the phloem bundles, more or less reticulated sclerenchyma cells are present (Plate 5*a* and *b*). It is not clear whether these cells belong to the cortex or to the central cylinder, and, in the latter case, whether they are part of the pericambium or of the phloem. The circumstance that the cells in question are situated exclusively at the periphery of the phloem bundles argues for the latter possibility. Close to the main central cylinder, lignification of pith cells takes place. This is also the case for those cells of the interfascicular regions which lie on the secondary xylem side.

Details of changes observed in the cortical region will be discussed in the next paragraph.

Secondary changes in the cortex

An increase in diameter of the central cylinder by the formation of secondary tissues will of course cause changes in the cortical as well as in the epidermal regions of the cladodes. Furthermore, these changes will be conspicuous where the formation of secondary xylem and phloem is at its highest. It must be stressed that this type of change is not restricted to flowering areoles but also takes place, to a more moderate extent to be sure, in the case of resting areoles.

There appeared, however, to be a second type of secondary change restricted to flowering areoles and taking place prior to the other type. Originally, the two types of cortex cells, i.e. chlorenchyma cells and mucilage cells, are both isodiametric in shape. The changes run along two lines: regular expansion in the case of flowering areoles only, on the one hand, tangential deformation and dilation caused by cambial activity in both flowering and resting areoles on the other. The mucilage cells especially exhibit considerable expansion (compare Plates 3*a* and 3*b*). Later on, in those areas where the formation of secondary vascular tissues takes place to a higher degree, the mucilage cells become deformed tangentially. True dilatation by cell division, however, is only rarely encountered (Plate 4*b*).

Expansion of chlorenchyma cells occurs to a lesser degree. Deformation in a tangential sense and dilatation may take place to a very considerable extent. As a matter of fact, even in those areas where no secondary growth occurs tangential deformation of some cells and a few cell-divisions can already be observed. (Plate 3*a*). These phenomena occur on a large scale where considerable amounts of secondary xylem and phloem have been formed. (Plate 4*b*). In such cases practically the whole cortex is subject to the transformations described.

Quantitative data

From cladode fragments containing vascular strands leading to resting and flowering areoles, several series of sections were prepared in such a way that branch and leaf traces were cut transversely. By means of a camera lucida, drawings were made on squared paper of sections of branch traces from certain measured distances from the midrib. Outline drawings were prepared of phloem and xylem, and of the sclerenchymatous tissue situated at the periphery of the phloem. The numbers of squares (mm^2) of the areas covered by these tissues were calculated and plotted graphically (Fig. 1).

From the data presented it is clear that branch traces of resting and flowering areoles differ greatly as to their rate of development. On the other hand, no fundamental differences could be observed. In the case of resting areoles, branch traces show more or less gradual increase in the amounts of phloem and xylem along the line from midrib to areole. In the case of flowering areoles, however, the amount

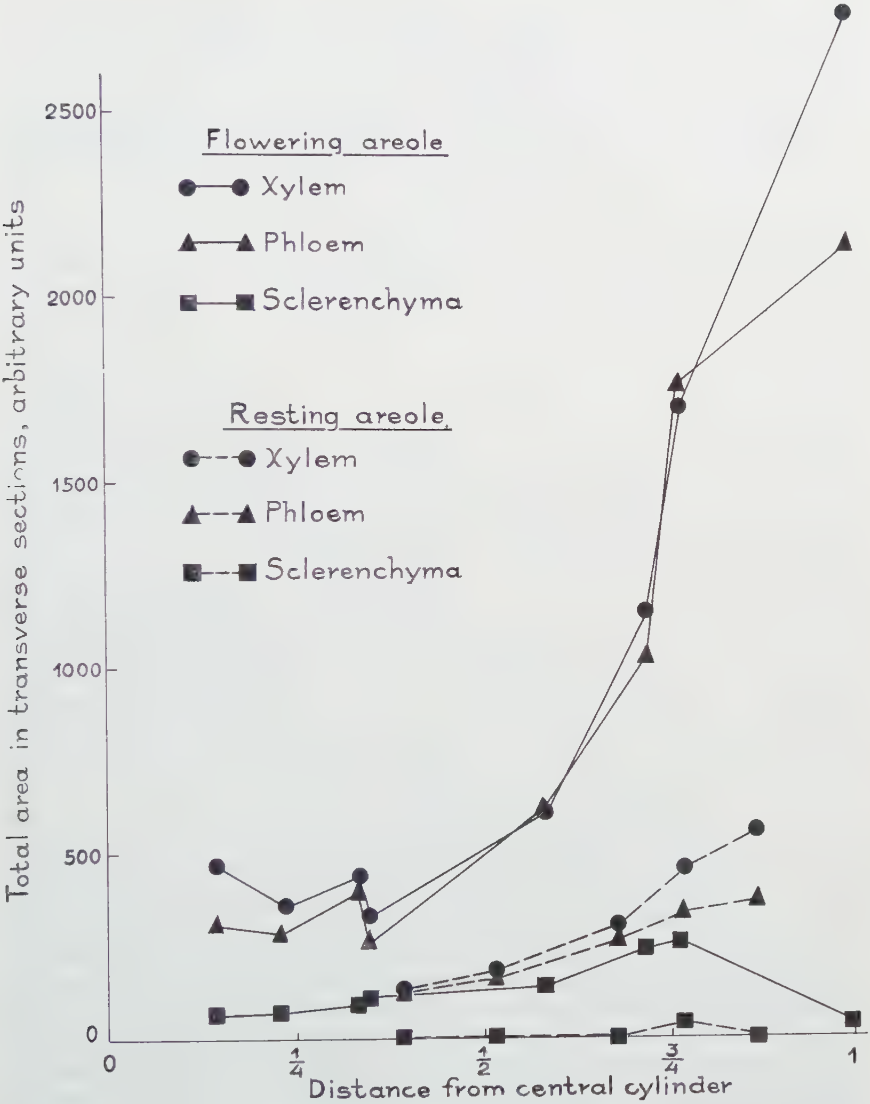

Fig. 1. Total areas of xylem, phloem, and sclerenchyma in transverse sections of branch traces of resting and flowering areoles of *Phyllocactus* at different distances from the central cylinder of the cladode.

of secondary phloem and xylem is very dramatically increased towards the areole.

As to the sclerenchyma, in the case of resting areoles only a very slight amount was encountered at a point about three quarters of the distance between midrib and areole. For the rest, no such sclerenchyma could be observed in this category. In the case of flowering

areoles, however, the amount of sclerenchyma is appreciable and to be found everywhere along the whole range. It is striking that the amount of sclerenchyma shows a maximum at the same point as in traces leading to resting areoles.

Some data can also be given on the relationship of cortex and central cylinder in branch traces of *Phyllocactus*-cladodes. From a

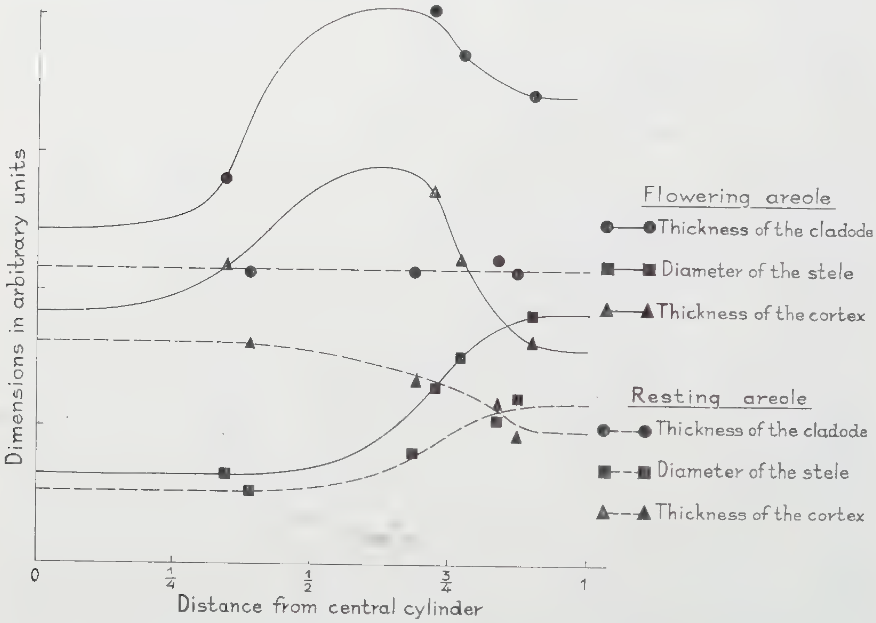

Fig. 2. Variations in dimension of some composing zones of *Phyllocactus* cladodes in relation to flowering.

series of photographs, values were obtained for several items, i.e. the thickness of the cladode along the course of the strands, the diameter of the stelar structure and the thickness of the cortex. Expressed in arbitrary units, the data are presented in Fig. 2. It can be clearly seen that in the case of resting areoles the thickness of the cladode itself remains constant from midrib to margin. This is remarkable because the diameter of the branch trace increases in diameter towards the margin. The only way by which a constant thickness of the cladode can be effected is obviously by a decrease of the dimensions of the cortical portions. This is indeed the case, as the radial dimensions of the cortical cells become reduced by the tangential stress roused by the expansion of the stelar structure. In the case of flowering areoles, the situation is fundamentally different which was to be expected from the previously described expansion of cortical cells in such cases. At a point about two thirds of the distance from midrib to margin, the cladode is at its thickest, while from this point toward the areole a slight decrease in thicknes can be observed. This

variation is the result of a number of successive and simultaneous processes. The increase of the thickness is for the greater part due to the already-mentioned expansion of the cortical cells. Later on, and this is also true in a topographical sense, a decrease in total thickness of the cladode occurs. This is only possible by a drastic decrease in radial sense of the cortical region, as in this part of the cladode the traces exhibit an important increase in diameter. This radial decrease of the cortex was indeed observed. It is due to the tangential stress caused by the greatly increased diameter of the central cylinder. Because of the fact that practically all cortical cells of the area in question are subject to this new change in shape, the resulting decrease of the whole cortex succesfully counteracts the increase of the dimension of the traces.

Discussion

From the data it seems obvious that developing flower-buds exert stimulating effects on certain tissues of *Phyllocactus*-cladodes which result in swollen strands running from the central cylinder of the cladode to the areoles. In the first instance this becomes evident by an expansion of those cortex cells which are situated around the vascular strand connecting the areoles from which flower-buds develop with the central cylinder of the cladode.

A second effect is encountered in the stimulation of cambial activity observed in leaf and branch traces of flowering areoles. As a result, considerable amounts of secondary xylem and phloem are formed. The formation of secondary tissues also takes place in traces leading to resting areoles but to a much lesser extent. The formation of secondary tissues in its turn causes new changes in the cortical region, which result in a decrease of the thickness of that region.

The external visibility of the strands is often heightened by the formation of considerable amounts of anthocyanins. The presence of these pigments suggests a raised sugar level, a phenomenon which has often been described (e.g. KARSTENS, 1938). In the present case it is understandable that notable amounts of different substances, a.o. sugar and water, have to be transported in order to supply the growing flower-buds with sufficient material. The observed differences in the amounts of phloem and xylem along the branch traces from the central cylinder to the areoles give, however, a somewhat astonishing impression as to the suitability of the transport system. For, there is a considerable discrepancy between the amounts of phloem and xylem in the vascular bundles just below the areole and those close to the main vascular cylinder, i.e. 7:1. This forms a bottleneck close to the main cylinder of the cladode. It seems, therefore, rather doubtful whether it is justified to consider the formation of vascular tissue as a provision to promote transport to the growing flower-buds.

With the results of SNOW (1935), GOUWENTAK (1936, 1941) and GOUWENTAK and MAAS (1940) in mind, the possibility of an activation of cytokinetic processes of the cambium by the developing flower-bud was considered. This is the more likely because of the fact that

leaf traces and anastomosing bundles of flowering areoles also exhibit secondary growth. Some experiments using lanolin paste containing growth substances to replace excised resting areoles did not lead to any stimulation of cytokinetic activities of the cambium. The thickening obtained adjacent to the application of the hormone paste proved to be caused only by a localized multiplication of cortical cells. Many of these newly formed cells, arranged in neat rows, are very much enlarged. Since they contain appreciable amounts of mucilage they greatly resemble the slime idioblasts described above.

As to the failure to induce cambial activity, the possibility remains that we have to do with a case comparable to that described by Gouwentak and Maas (1940) and Gouwentak (1941). According to these authors cambium must be roused from dormancy before growth hormones can stimulate cytokinesis. It is our intention to continue our experiments with this point in mind.

SUMMARY

Phyllocactus plants coming into flower exhibit thickened, often purplish coloured strands running from the central vascular system of the cladode to the budding areoles. The anatomical background of this phenomenon forms the major part of the present paper. It appears that the growing flower-bud stimulates certain tissues of *Phyllocactus* cladodes, resulting in the formation of swollen strands. This stimulation successively affects two types of tissue, i.e. the parenchymatous cortex surrounding the vascular traces connecting the central cylinder of the cladode with the areole and, secondly, the cambial zone of the vascular bundles present in such traces. The cortex parenchyma as a result exhibits a general expansion of the cells, and the cambial zone is stimulated to form considerable amounts of secondary tissue. The production of these tissues causes tangential stretching of the surrounding cortical cells which results in a decrease in their radial dimension. In the proximal part of the strands, cell expansion proved to be the major component of the swelling; in the distal part this is caused preponderantly by the formation of secondary vascular tissues.

There is a marked difference in the quantities of vascular tissue formed along the strand. A ratio of 7:1 was observed on comparison of distal and proximal portions of branch traces.

Some introductory experiments with lanolin paste containing growth substances to replace excised resting areoles produced negative results. These investigations will be continued.

ACKNOWLEDGEMENTS

Much appreciation is due to Mr P. D. Burggraaf for microtechnical advise, to Mrs E. F. van Balen–Meynema, formerly attached to our laboratory, and to Miss H. W. Doornberg for the preparation of the photographs, and to Mr. J. E. Bevelander for the execution of the drawings.

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Plate 1*a*. *Phyllocactus*. Cladode with a number of flower-buds in various stages of development. The "midrib" and the thickened strands towards the areoles are clearly visible. ± nat. size.

Plate 1*b*. Epidermis and cortex of *Phyllocactus* cladode. A, epidermis with cuticle and stoma; B, hypodermis; C, chlorenchyma cell; D, mucilage idioblast. 200 ×.

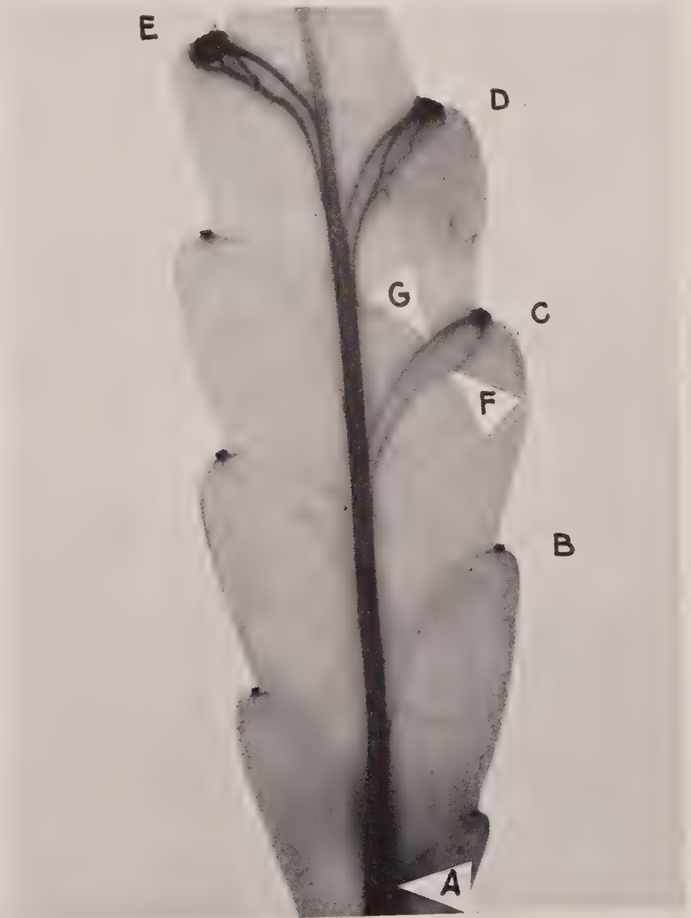

Plate 2. Vascular system of *Phyllocactus* cladode, decolourized and made transparent. Flowers and flower-buds removed. A, central cylinder ("midrib") of the cladode; B, resting areole; C, place of insertion of a one-inch long flower-bud; D, ditto of flower-bud 4.5 inches long; E, ditto of fully expanded flower; F, leaf trace; G, branch trace. Anastomoses between leaf and branch traces are clearly visible. Somewhat larger than natural size.

Plate 3*a*. *Phyllocactus*. Resting areole. Section perpendicular to the long axis of a branch trace close to the central cylinder of the cladode. A, branch trace; B, mucilage idioblast; C, first indication of dilatation in the cortical region. 35 ×.

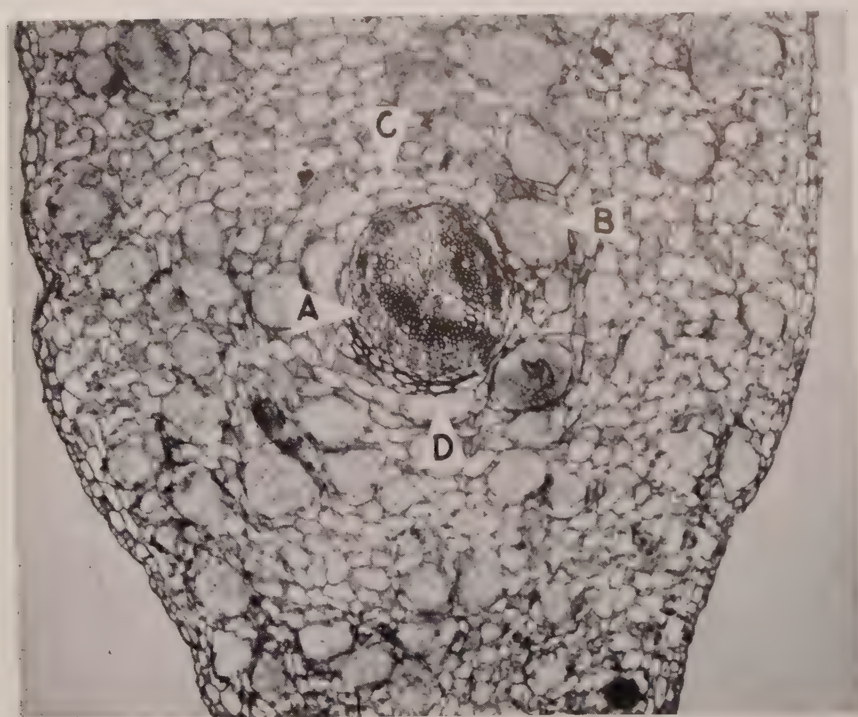

Plate 3*b*. *Phyllocactus*. Flowering areole. Section as in Plate 3*a*. A, branch trace; B, mucilage idioblast; C, dilatation of cortex cells; D, sclerenchyma. 35 ×.

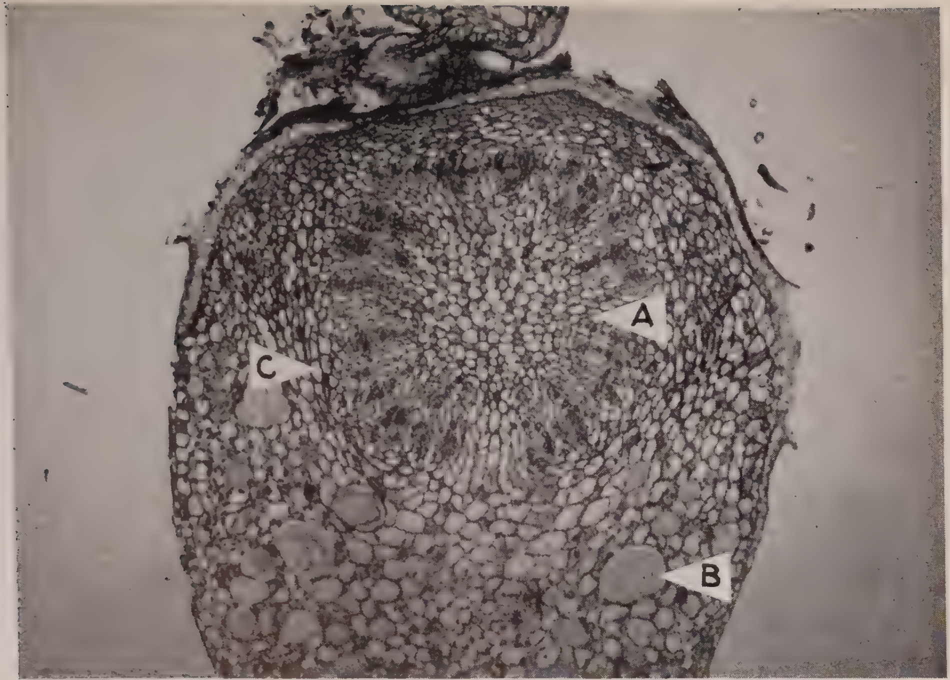

Plate 4*a*. *Phyllocactus*. Resting areole. Section perpendicular to the long axis of a branch trace close to the areole. Several cortical layers show dilitation. A, branch trace; B, mucilage idioblast; C, dilatation of the cortex. 35 ×.

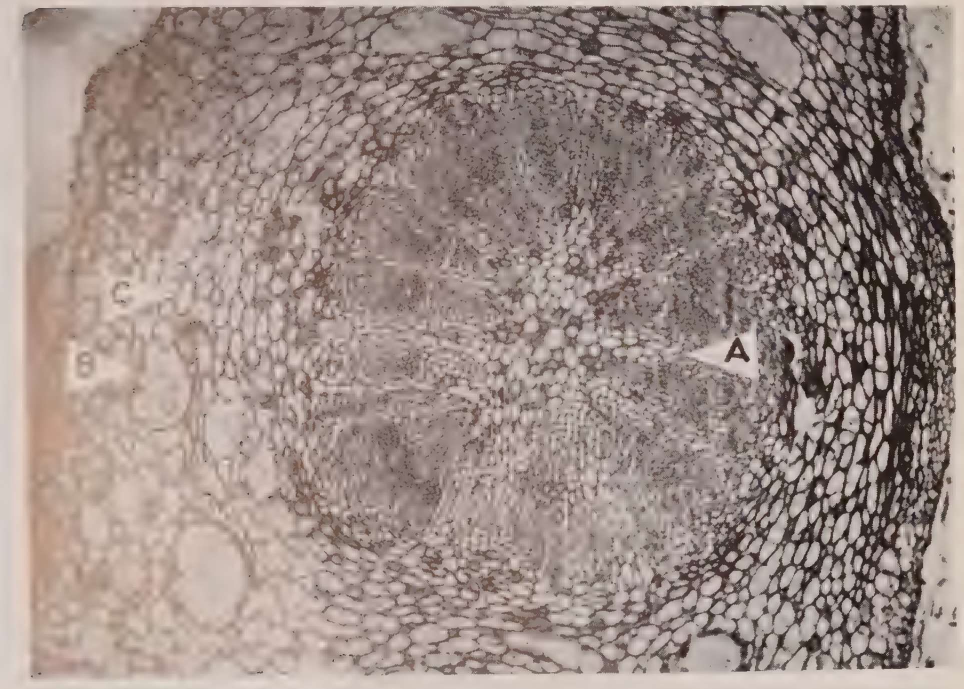

Plate 4*b*. *Phyllocactus*. Flowering areole. Section as in Plate 4*a*. Many cortical layers show dilatation. A, branch trace; B, dilatating mucilage idioblast; C, dilatation of the cortex. 35 ×.

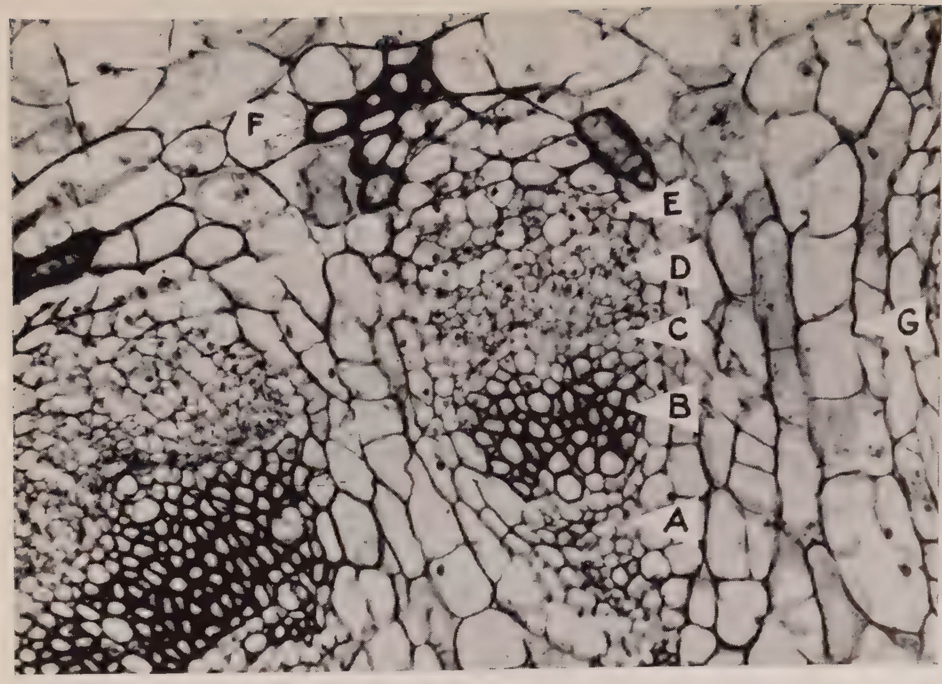

Plate 5*a*. *Phyllocactus*. Flowering areole. Detail of a branch trace in transverse section. A, primary xylem; B, secondary xylem; C, fascicular cambium; D, secondary phloem; E, primary phloem; F, sclerenchyma; G, interfascicular region composed of irregular rows of radially orientated cells. At the top left, many dilatating cortical cells. 150 ×.

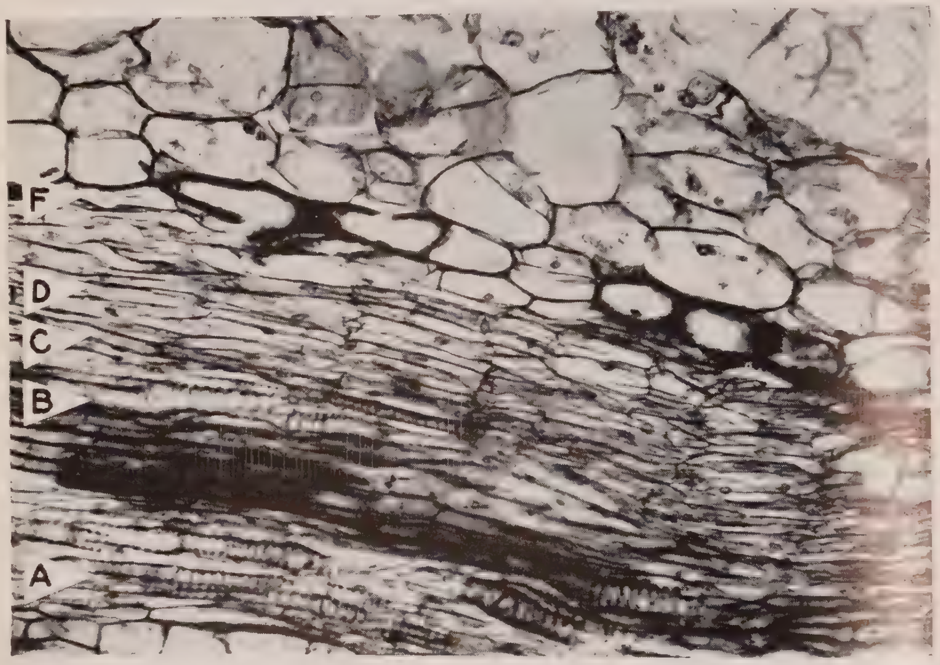

Plate 5*b*. *Phyllocactus*. Flowering areole. Detail of a branch trace in longitudinal section. A, primary xylem; B, secondary xylem; C, cambial zone; D, secondary phloem; F, sclerenchyma. Primary phloem indistinct. At the top right, part of a mucilage idioblast is visible. 150 ×.

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BRIEF REPORTS

JONKER, F. P.: Hypericum canadense in Europe: an Addition

In the previous volume of this periodical the present author reported on the occurrence of *Hypericum canadense* L. in the Netherlands, together with that in a similar locality in Ireland and in a more deviating locality in France, discovered by WEBB and by BOUCHARD respectively.

Dr. C. SIMON of Basel, Switzerland, was so kind to send dried material of two collections from the French locality, collected by him on August 17th, 1958, and August 20th, 1959 (Haute Saône, at the beach of a lake in the region of the Etang d'Arfin, near La Mer, alt. 540 m). This material, however, appeared to belong to another species, though related to *H. canadense*, namely, *Hypericum majus* (A. Gray) Britt. Dr. Simon himself arrived already at the same conclusion, which, according to his letter, was confirmed moreover by Dr. MERXMUELLER who reported, in 1956, the finding of the latter species in Germany, in a locality where it had been introduced by U.S. military units during world war II. It is remarkable in this connection that BOUCHARD in his discussion of the possibilities of introduction into France mentioned the stay in the area in question of U.S. army units during world war I.

It is clear, at any rate, that the record of the species as occurring in France is due to a misidentification. Nevertheless the question remains whether the species is to be considered a relic in Europe.

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In this paper all other literature was cited.

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BOOK REVIEWS

OF PUBLICATIONS RELATED TO BOTANICAL WORK IN THE NETHERLANDS

Robert B. Withrow (editor) — Photoperiodism and Related Phenomena in Plants and Animals. Publ. no 55 of the Am. Ass. for the Adv. of Sc. Washington D.C. 1959.

Dit boek is een verslag van hetgeen verteld werd op de "Conference on Photoperiodism", gehouden van Oct. 29 – Nov. 2 1957 in Gatlinburg, Tenn. en georganiseerd door de Committee on Photobiology of the National Academy of Sciences – National Research Council. Het voornaamste doel was het scheppen van een discussie-gelegenheid waar de facetten van de fotoperiodiciteit zowel van de botanische als van de zoölogische kant belicht konden worden. Het spreekt vanzelf, dat een dergelijk symposium een zeer groot aantal voordrachten uitlokte, waardoor het niet goed mogelijk is een volledig overzicht over het geheel ervan in enkele bladzijden weer te geven.

De eerste groep voordrachten gaat over problemen van foto-chemische aard en over de primaire fotochemische reacties. Een inleiding van Oster geeft een overzicht van fotochemische reacties van kleurstoffen. Hij geeft hierbij een groot aantal voorbeelden van fotoreducties en fotooxidaties doch blijft daarbij uitsluitend op zuiver chemisch gebied, dus op het gebied van de ongeorganiseerde systemen.

Daarna behandelt French het verband tussen actie-spectra en absorbtie-spectra van de pigmenten in de cel en wijst daarbij op de moeilijkheid van het meten van een absorbtiespectrum. Het pigment zal in oplossing vrijwel altijd een ander absorbtiespectrum hebben dan in de cel. Daar zijn de pigmenten gewoonlijk gebonden aan grote moleculen; bovendien zal de verstrooiing van het licht het meten sterk bemoeilijken terwijl bovendien nog de verstrooiing door gekleurde deeltjes weer gecompliceerder is dan die door ongekleurde deeltjes. Dit effect wordt vaak te weinig gerealiseerd door de onderzoekers.

Brackett en Hollaender geven nog een beschouwing over actie-spectra, terwijl Tollin, Sogo en Calvin de energie overdracht in geordende en ongeordende systemen bespreken.

Dan volgt een tweede groep voordrachten, waarin het onderwerp photoperiodiciteit naar voren komt. Wareing vond dat verschillende lichtgevoelige zaden bij een bepaalde temperatuur reageerden op de daglengte. Zo kiemt bijv. zaad van *Nemophila insignis* bij 21 a 22° C slechts in korte dagen. Bij lichtgevoelige zaden is bekend dat zij zeer duidelijk het antagonisme rood-infrarood te zien geven. Wareing constateerde bij zaad van Grand Rapids sla ook een rood-blauw antagonisme, waarbij blauw licht echter lang niet zo sterk werkzaam was als infrarood. Bij het bestuderen van de winterrust bij *Acer pseudoplatanus* die door korte dagen wordt geinduceerd bleek dat onder invloed van korte dagen meer remstof in de bladeren werd gevormd dan in lange dagen.

Toole geeft daarna een overzicht van een groot aantal plantensoorten waar zaden lichtgevoelig bleken te zijn. Ook het rood-infrarood antagonisme wordt uitgebreid door hem besproken. Meyer geeft dan een bespreking van proeven met *Salvia occidentalis* opgegroeid in verschillende spectrale gebieden onder verschillende daglengten. Hierbij kwam het interessante verschijnsel naar voren dat een plant

door de lichtkleur van de korte dag klaar gemaakt moet worden om te kunnen reageren op stoorlicht. Het bleek dat blauw licht in dit opzicht zeer actief is, rood licht en groen licht practisch inactief zijn. Een weinig infrarood aan het groene licht toegevoegd maakt deze combinatie echter zeer actief. Ook hier kwam dus weer een overeenkomst in werking tussen blauw en infrarood naar voren. WASSINK en DE LINT bespreken het gedrag van *Hyoscyamus niger* en van sla als deze planten in verschillend gekleurd licht opgroeien, waarbij zij wijzen op de overeenkomst tussen de invloed van gibberellazuur en een bestraling met blauw of infrarood. DOWNS gaat speciaal in op het rood-infrarood antagonisme. De biochemie van dit antagonisme is het onderwerp van GALSTON die bij erwten een stof kan isoleren die de IAA-oxidase remt. Hoe langer de dag is, des te meer van deze oxidase-remmer wordt er gevormd.

Hierna volgt een serie voordrachten over een mogelijk verband tussen toegevoegde chemicalien en de invloed van het licht. Zo passeren telkens weer groeihormonen, gibberellazuur, kinetine, FMN en cobalt de revue, in hun interactie met de daglengte-invloeden. Het heeft geen zin om hier alle auteurs te noemen; een groot aantal feiten wordt gegeven, een grote lijn is er echter nog niet duidelijk uit te halen.

Een analyse van het proces der fotoperiodiciteit door BONNER wijst op de gecompliceerdheid van dit proces. Anton LANG bespreekt de invloed van gibberellazuur op de bloei en CATHEY komt dan met een antagonist van gibberellazuur, het Amo-1618, een quaternaire ammoniumverbinding. Zowel BORTHWICK als HENDRICKS geven een analyse van het rood-infrarood antagonisme, waarbij de conclusie luidt dat men te doen moet hebben met een reversibele fotoreductie, waarbij de physiologische activiteit gekoppeld is aan de infrarood absorberende toestand van het pigment. Een kinetische analyse van de fotoperiodiciteit door WITHROW leidt tot een gecompliceerd schema, waarbij op deze conferentie het bestaan van een inwendige klok voor het eerst haar intrede doet.

Hiermede begint een serie voordrachten over dit inwendige ritme, waarbij de zoölogen zich niet onbetuigd laten. PITTENDRIGH en BRUCE geven een aantal voorbeelden van dieren en planten, die een uitgesproken 24 uursritme vertonen. We treffen er zowel zoogdieren als insecten aan naast schimmels en algen. Men ontkomt dus niet aan de indruk dat het hierbij gaat om een zeer universeel mechanisme. Om een theoretisch model te bedenken dat deze verschijnselen zou kunnen vertonen, komen zij tot een aanname van tenminste twee oscillatoren in de cel die onderling gekoppeld zijn en waarvan de een lichtgevoelig en de ander temperatuurgevoelig is. BÜNNING gaat verder op hetzelfde onderwerp door en vult het aantal voorbeelden van ritmische verschijnselen aan met een aantal in het gebied van de physiologie der hogere planten. Ook WENT geeft meer voorbeelden. HASTINGS en SWEENEY bespreken proeven met *Gonyaulax*, een Dinoflagellaat die het lichten van de zee kan veroorzaken. Dit algje fosforesceert bij prikkeling, doch doet dat alleen gedurende 12 van elke 24 uren, ook als het onder constante omstandigheden wordt opgekweekt. Ook hier dus een inwendige klok. Deze klok kan gelijk gezet worden door licht.

Verder volgen er nog een aantal auteurs die steeds meer voorbeelden aandragen van dieren en planten met inwendige klokken, terwijl de theoretische beschouwingen er aan vastgeknoopt steeds ingewikkelder worden. Kennelijk tast men bij dit nieuwe verschijnsel nog geheel in het duister als men de oorzaken er van tracht te vinden.

Naast de besprekingen van de inwendige 24 uurs klok komen nog beschouwingen over een ingebouwd jaarritme naar voren.

In een hierop volgende serie mededelingen komt de echte fotoperiodiciteit weer naar voren. Lees geeft een overzicht over de invloed van de daglengte op insecten en mijten. Zowel het uitkomen der eieren als het in diapauze gaan wordt door hem met vele voorbeelden toegelicht. Giese geeft voorbeelden van Echinodermen en Mollusken die voor hun voortplanting van de daglengte afhankelijk blijken te zijn, terwijl Bullough en enkele anderen dit doen in de groep der Vertebraten. Vooral bij de vogels komt er een zeer duidelijke fotoperiodiciteit te voorschijn.

Als slot komt men in een lange mededeling van Halberg, Barnum en Bittner weer terug op de inwendige klok, doch thans bij mensen en muizen. Zij komen tot de conclusie dat vele fysiologische verschijnselen een eigen 24 uurs-ritme vertonen, doch dat deze allen een duidelijke neiging hebben om synchroom te verlopen.

Wie het gehele boek doorleest, (meer dan 850 pag.) zal zeker niet de indruk krijgen dat de oplossing van de problemen van fotoperiodiciteit en inwendige klokken nabij is. Vermoedelijk zal de lezer een zeer gecompliceerd en misschien wel verward beeld overhouden.

Voor diegenen echter, die op de hoogte willen komen van de stand van de wetenschap op de bovengenoemde gebieden is het bestuderen van dit werk sterk aan te bevelen. Er is een schat van materiaal bijeengebracht door talloze onderzoekers die op dit gebied werken. Duidelijk komt naar voren, dat de onderwerpen nog in het stadium zijn waarin elk onderzoek slechts nieuwe problemen bijdraagt in plaats van dat het raadselen oplost. Voor de specialist is het boek onmisbaar omdat nergens zoveel materiaal is bijeengebracht.

Als tragische bijzonderheid zij nog vermeld dat het boek gewijd werd aan de nagedachtenis van Dr Robert Withrow, een van de meest actieve organisatoren van de conferentie. Door zijn ziekte kon hij slechts het eerste gedeelte van de conferentie meemaken. Tijdens het prepareren van de manuscripten overleed Withrow, zodat hij helaas het werk niet meer heeft zien gereed komen. Ongetwijfeld is het welslagen van de conferentie voor een zeer groot gedeelte aan hem te danken geweest.

R. v. d. V.

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THE PARASITISM OF EXOBASIDIUM JAPONICUM SHIR. ON AZALEA

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CONTENTS

CHAPTER I

INTRODUCTION

The growing of Azaleas plays an important part in many horticultural centres of Western Europe, such as Dresden in Germany, Ghent in Belgium, and Boskoop in the Netherlands. Every year

Dutch growers spend large sums for buying Azaleas, mostly young plants, from the Belgian horticulturists. In the Dutch nurseries these plants are grown until they begin to flower, and are ready to be sold. The large assortment of Azaleas that is grown under the names *Azalea indica* and *Azalea obtusa*, consists of a great variety of cultivars which, botanically speaking, all belong to the *Rhododendron* group.

Often these plants suffer from a disease known in Dutch as "oortjes ziekte" or "ezelsoren", indicating that the deformed leaves are shaped like ears. The diseased leaves are greatly enlarged and thicker than normal, the leafblades becoming convex and crooked because of irregular growth. The original shape of the leaves can no longer be recognized, and it is understandable that these misshapes reminded people of ears (Plate 1, A and B). In this article these hypertrophic organs will be called galls.

The disease is caused by a fungus known as *Exobasidium japonicum* Shir. and belonging to the *Basidiomycetes*. The mycelium of the fungus present in the intercellular spaces of the leaf tissue, produces the hymenium, which develops under the epidermis, and consists of a layer of cells, the basidia. After breaking through the epidermal cells, the basidia form basidiospores, and as the latter are produced in enormous numbers, they give the galls a velvety appearance. The colour of the diseased parts becomes white to reddish at that stage. The galls develop gradually, often unobserved, as they are frequently hidden under flowers and leaves. When the flowering period is over, they seem to appear suddenly, to the surprise of the owner of the Azalea, who thought himself in possession of a healthy plant. This disease was observed for the first time in Western Europe at the turn of this century. It is possible that it was imported with plants from Japan, for the fungus was already described in that country by Shirai (1896).

The lifecycle of *Exobasidium* on Azalea is only incompletely known. Thus, the first purpose of the experiments described in this paper, was to obtain better information concerning the nature of the parasitism of this fungus. An answer to the following questions was still lacking: at what stage of the development of the plant does the parasite penetrate and under which circumstances; is one basidiospore sufficient to produce an infection, or is the fungus heterothallic?

Another question to be answered was how the length of the incubation period varies under different conditions. The presence of the symptoms can easily be observed, but the moment of infection is as yet unknown. Therefore, it is difficult for the growers to know when preventive measures have to be carried out. Some experiments were carried out with a small number of fungicides, which were applied before and after the Azaleas were inoculated with the parasite. This kind of research, however, will have to be continued in large-scale cultures.

In this paper some attention is also paid to the nomenclature of the Azalea parasite. Shirai (1896) has been the first to describe an *Exobasidium* on the 'Indian Azalea'. American mycologists described

parasites on wild *Rhododendron* species and on other members of the *Ericaceae* family occurring in North America as new species of the genus *Exobasidium*. It is still an open question whether all these species are distinct, or whether the parasites described by Shirai and the American authors are identical with *Exobasidium vaccinii* named and described by WORONIN in 1867. If the various species should prove to be conspecific, this name has priority. Many authors are of opinion that they actually are conspecific, as no morphological distinction can be found between the parasites living on different host plants. The results of cross inoculations with *Exobasidium* spores of different origin would enable us to answer the question whether these parasites are fully identical, or whether they show specialization on their host plants. To that purpose a collection of host plants with their parasites is indispensable. The experiments described in this paper were, however, restricted to cross inoculations with *Exobasidium* originating from Azalea and from *Vaccinium vitis idaea*. This *Vaccinium* and some Azalea cultivars were used as host plants.

The purpose of this investigation was to obtain an answer to the aforesaid questions concerning the life cycle of the *Exobasidium* parasite on Azalea, and to investigate whether this fungus is identical with that described by Woronin. Finally, the striking similarity between the parasitism of *Exobasidium* spp. and that shown by *Taphrina* spp., belonging to the *Ascomycetes*, will be discussed.

CHAPTER II

TAXONOMY AND CULTIVATION OF AZALEAS

Linnaeus described in his Species Plantarum (1753) the red Alpine Rose, which he named *Rhododendron ferrugineum*. This, therefore, is the type of the genus *Rhododendron*.

About a century later Hooker's expedition to the Himalayas brought 45 new *Rhododendron* species to Western Europe, which were described in 1851. More new species were introduced into Europe from America as well as from Western China, Japan, Burma, Indo-China, Malaya and other Asiatic countries. At present about 800 to 900 species are known, including hardy ones, called *Rhododendron* and non-hardy ones, which shed their leaves under unfavourable conditions. The latter were placed in the genus *Azalea*. However, as hybridization between more and less hardy species is easy, it is not justified to retain *Azalea* as a distinct genus. It should be sunk into *Rhododendron*. The latter can be divided into two subgenera: *Eurhododendron* and *Anthodendron*. To the former are assigned the *Rhododendron* species bearing leathery leaves which are not shed during winter. In the subgenus *Anthodendron* are placed about 70 non-hardy, soft-leaved species, which in practice are called Azalea. As the name Azalea is generally used by the growers, the name will be used in this paper too. All experiments were performed with *Rhododendron* plants of this type. The Japanese Azaleas may be assigned to the 'Tsutsutsi' section

of the subgenus *Anthodendron*. They are also called 'Kurume' Azaleas. *Rhododendron obtusum* Planch. was obtained by a crossing of the hardy *Rhododendron kaempferi*, originating from the Japanese plains, with other Japanese species. The grower W. F. Koppeschaar obtained the small- and pink-flowered cultivar 'Esmeralda' from Japanese seed. Other cultivars grown at the Boskoop centre of horticulture are 'Galestin' and 'Moederkensdag'. They all belong to the 'Kurume' group, and are designated by the growers as *Azalea obtusa*. The Indian Azalea has *Rhododendron simsii* Planch. as ancestor, and is called by the growers *Azalea indica*. A hybrid of this species and a *Rhododendron obtusum* var. *amoenum* is the cultivar 'Hexe' frequently grown in Belgian nurseries (GROOTENDORST, 1954; ENCKE, 1959).

For the cultivation of Azaleas soil is used from the oak and fir woods of the "Campine", a region in the northern part of Belgium. The plants are grown from seed or from cuttings. When 8 months old, the "baby Azaleas" are in possession of 6 to 8 leaves, and at the age of 18 months they can be sold. The cuttings are obtained by trimming the plants either in August to September, or during the period between February and April. The humidity of the air in the rooting beds has to be high. Young plants, i.e. plants which have to be kept in the nursery for a second year before they start flowering, are frequently bought by Dutch growers from Belgian firms. The value of the *Azalea indica* imports from Belgium and Luxembourg into the Netherlands in 1957 was estimated at about 1.2 million Dutch guilders (VANDENDAEL, 1960). In that year, according to the "Tuinbouwgids", 1960 (Dutch Horticultural Guide), 540.000 *Azalea indica* and *Azalea obtusa* plants were sold at the Dutch auctions.

The plants are not hardy under our climatological conditions. Most varieties react on an exposure to night frost by shedding their leaves. Thus they have to be kept in the greenhouses till the beginning of May, when they are brought into the open (VAN RAALTE, 1955). However, protection against late night frosts is still necessary. They are densely planted in the beds. In autumn they have to be returned to the greenhouse. During autumn and winter they are exposed to a temperature of about 16° C, which may be raised gradually at the time of the development of the flower buds. However, if the temperature becomes too high, which may occur in early spring, the doors of the greenhouses are opened in order to lower the temperature. In all cases a high humidity of the air and a high moisture content of the soil are conditions for good development. Therefore, the plants are continuously sprayed, often by means of mist sprayers, during the time the plants are outside as well as during the time they are kept in the glasshouses. This spraying and the opening of the glasshouse doors in order to lower the temperature are the cause of heavy air-currents blowing through the glasshouses. In this way microorganisms, including spores of pathogens such as *Exobasidium japonicum*, can be spread easily from diseased to healthy plants. The high humidity of the air keeps the spores viable and favours infection.

CHAPTER III

OCCURRENCE OF THE DISEASE

In the centra of the Azalea cultivation the *Exobasidium* disease, already known for over half a century still occurs frequently. Susceptible varieties, such as 'Hexe', may be diseased to such an extent that the same plant may seem to carry besides its normal red flowers also somewhat deformed white ones. The latter are the many big galls covered with enormous amounts of spores. Though the galls appear throughout the year, gall formation has an optimum in certain months. An inquiry among the Belgian growers revealed that galls are most frequent during March and April; at this time the flowering period is finished, but the plants are still kept in the greenhouses. During June galls may be frequent on plants in the open, and sometimes this may be so in September too (WELVAERT, 1952). One gets the impression that most of the galls appear at the time the plants are resuming their vegetative development. This occurs after flowering, when underneath the whithering flowers the axillary buds begin to develop. Renewed vegetative growth also occurs some weeks after the plants have been moved to the outside, i.e. in June. It has become clear from these facts as well as from our own observations that galls may appear as the buds start to unfold. The young galls become visible as small, somewhat swollen, translucent spots on the young leaves. By hypertrophic growth they soon become more conspicuous. They turn into galls of an irregular shape, and with a light green colour and a smooth, shining surface. When the fungus starts sporulating, the basidia rupture the cuticle, and the basidiospores are formed. At that time the surface of the galls gets a powdery aspect, and the colour changes from light green to snow-white. On account of the development of secundary spores sprouting from the basidiospores, the surface of the galls subsequently becomes covered with clusters of these spores, for which, as will be expounded here after, the name blastospores will be used; this gives them a velvety aspect. The diseased tissues then rapidly disintegrate: the galls shrivel, become brown and soon fall prey to all sorts of saprophytes. The parasite may attack all parts of the plant except root tissue. Stems and leaves as well as flower parts, therefore, may show hypertrophic growth caused by the parasite. Most galls, however, occur on the leaves, which usually are only partially attacked. The hypertrophic part is sharply separated from the healthy leaf tissue.

From these observations it was assumed that a bud can be infected when still in a dormant state. This hypothesis is in agreement with the observation that young galls are never present on fully developed leaves. The galls always appear simultaneously with the expanding of the young but still folded leaves of a developing bud. The resumption of growth by the dormant bud would coincide with a renewal of the activity of the pathogen. This would also explain the sudden appearance of the galls in plants which were apparently healthy

when imported. This mode of development would be in agreement with that described by WOLF and WOLF (1952) for *Exobasidium camelliae* var. *gracilis* Shirai on *Camellia sasanqua*.

CHAPTER IV

THE FUNGUS

4.1. REVIEW OF LITERATURE

The genus *Exobasidium* was proposed by WORONIN (1867), when he described the fungus causing a disease in *Vaccinium vitis idaea* as *Exobasidium vaccinii*. Though FUCKEL (1861) had observed the fungus already at an earlier date, this author did not give a good description of the pathogen, which he mistook for a *Fusidium*. After the appearance of Woronin's publication, many other *Exobasidium* species were described. Usually they were named after the host plants on which they occurred, but knowledge of their pathogenicity was lacking. The first *Exobasidium* occurring on a species of the genus *Rhododendron* was described by ELLIS (1874) as *Exobasidium discoideum*. It occurred on a North American wild species, viz. *Azalea viscosa*. Its basidiospores have a length of 20 μ. In the same year PECK (1874) described *Exobasidium azaleae* from the wild *Azalea nudiflora* growing in the same country. In this species the length of the basidiospores is 15–20 μ. Both authors paid much attention to the shape of the galls. SHIRAI (1896) described a parasite occurring on *Azalea indica* in Japan as *Exobasidium japonicum*. The length of the basidiospores of this parasite was 14–15 μ. RITZEMA BOS (1901) was the first to report an attack by *Exobasidium azaleae* Peck observed in a Dutch nursery in 1900. In a Belgian nursery an attack of *Azalea indica* was mentioned by LAUBERT (1909). This *Exobasidium*, which probably had been imported from Japan, would be identical either with *Exobasidium japonicum* Shir. or with *Exobasidium pentasporium* Shir. In Germany the disease was discovered in 1906 (NAUMANN, 1909). RACIBORSKI (1909) determined a parasite on *Rhododendron flavum* as *Exobasidium discoideum* Ellis. RICHARDS (1896) was already in doubt about the validity of many of the *Exobasidium* species created on account of their presence on different hosts, and often called after the latter. He could prove that *Exobasidium andromedae* Peck was identical with *Exobasidium vaccinii* Wor., as both parasites were able to cause similar disease symptoms on *Andromeda ligustrina*. In morphological respect several species prove to be indistinguishable. FARLOW (1877) considers *Exobasidium discoideum* Ellis and *Exobasidium azaleae* Peck as identical with *Exobasidium vaccinii* Wor., as no morphological differences between these species could be found. According to this author the names of the first mentioned species are superfluous, and only that of the last one should be retained.

NAUMANN (1909) was cautious in his conclusion concerning the identity of the Exobasidia occurring in Germany. He considers it to be impossible to identify the parasites on the base of their morpho-

logy alone. Many authors, however, agree with Farlow that the differences in size between the basidiospores of *Exobasidium vaccinii* Wor. and of several of the *Exobasidium* species that were described afterwards are so small that these parasites have to be considered as belonging to one species; the latter would comprise all *Exobasidium* species occurring on *Rhododendron* species (LIND, 1913; BURT, 1915; LAUBERT, 1925; MILES, 1928; and SAVILE, 1959). MARCHIONATTO (1929) too considers *Exobasidium vaccinii* Wor. the cause of galls occurring on Azaleas cultivated in Argentina. According to this author, *Exobasidium azaleae* Peck, *Exobasidium discoideum* Ellis and *Exobasidium rhododendri* Cramer are identical with *Exobasidium vaccinii* Wor. LAUBERT (1909 and 1932) retains for the parasite on Azalea the name *Exobasidium japonicum* Shir., though he remarks that the difference between this species and that described by Woronin is not clear. This also applies to *Exobasidium rhododendri* Cramer, a parasite on *Rhododendron ferrugineum* and *Rhododendron hirsutum*. However, according to this author, *Exobasidium pentasporium* Shir., which occurs on *Azalea indica* in Japan but not in Europe, seems to be another good species. SAVILE (1959), who studied the morphology of many *Exobasidium* species, is also of opinion that several parasites belonging to the genus *Exobasidium* have erroneously been described as new species. Several of them are identical. It is not excluded, however, that some of them may be regarded as formae speciales occurring on different host plants. Only experiments can give an answer to the question whether specialization exists or not. In this investigation cross inoculations were performed with *Exobasidium* spp. originating from Azalea and from *Vaccinium vitis idaea*.

4.2. DESCRIPTION OF THE FUNGUS ON AZALEA

The mycelium is intercellular. The width of the hyphae, $\pm 1\ \mu$, depends on the width of the intercellular spaces. The mycelium forms a subepidermal layer. The apices of the hyphae, the future basidia,

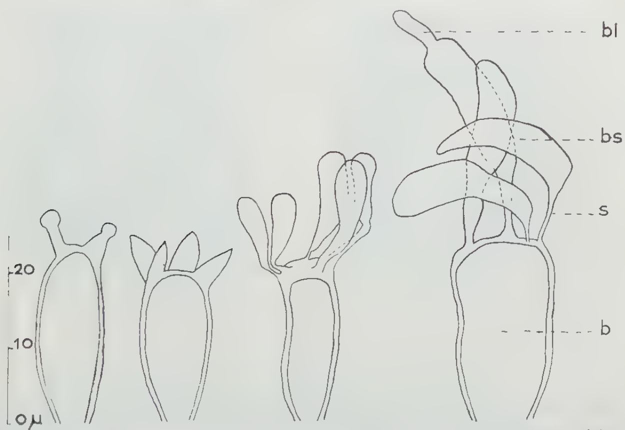

Fig. 1. Basidia (b) developing basidiospores (bs) on sterigmata (s). One of the basidiospores forms a blastospore (bl) in situ.

stretch in a direction perpendicular to the epidermis, thus forming the hymenium. By further growth the basidia find their way between the epidermal cells and rupture the cuticle. The basidia are cylindrical, 24–30 × 5–8 μ. The basidiospores develop on sterigmata, 4 to 5 on one basidium. The size of the basidiospores is 13–18 × 2.5–5.5 μ (Fig. 1). By budding the basidiospores produce a large number of spores, measuring 9–13 × 2–4 μ. They are designated as "conidia" by Woronin. According to Mix (1949), an analogy exists between the behaviour of the ascospores of the genus *Taphrina* and the basidiospores of the genus *Exobasidium*, in so far that both may produce new spores by budding. Mix uses the name "blastospores" for these products, which reminds one of the outgrowths formed by yeast cells. Among the *Protobasidiomycetes* too many forms occur, e.g. the smut fungi, in which the basidiospores produce new spores by budding. In this case the newly formed spores are called "sporidia" or "secondary sporidia". In this paper the yeast-like spores of *Exobasidium* will be called "blastospores".

4.3. Anatomy of the galls on azalea

Not only macroscopically (p. 351), but also microscopically a sharp boundary is seen between the healthy and the diseased tissue, when a gall is studied in transverse sections. There is a lack of differentiation in the mesophyll of the hypertrophic part, and tracheal elements are but sparingly found in the uniform tissue. In comparison with the

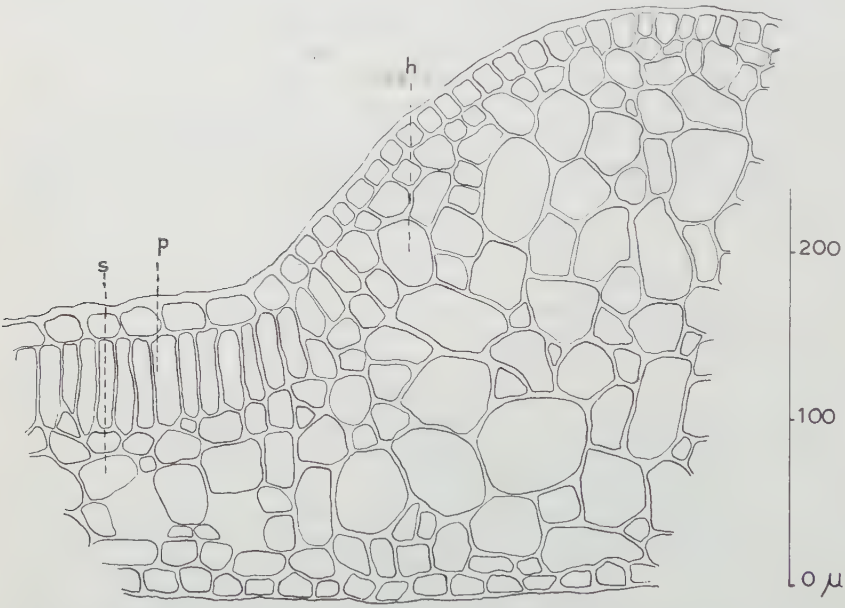

Fig. 2. Transverse section through an infected leaf, showing a healthy part with palissade parenchyma (p) and spongy parenchyma (s), and a diseased part with hypertrophic cells (h).

PLATE

Plate 1. A. *Azalea obtusa*, cultivar 'Esmeralda', with galls of *Exobasidium japonicum* Shir. B. *Azalea indica*, cultivar 'Hexe', with galls of *Exobasidium japonicum* Shir. C–G. Development of axillary buds of *Azalea indica*, cultivar 'Hexe', after decapitation of the shoot. C: just after decapitation; length of the buds about 2 mm. D–G: further developmental stages; length of the buds in D: 4 mm; in E: 6.5 mm; in F: 9 mm and in G: 12 mm.

normal mesophyll, the number of cells as well as their size have increased: both hyperplasy and hypertrophy occur (Fig. 2).

This same anatomical picture has been observed by several other authors (PETRI, 1907; NAUMANN, 1909; MARCHIONATTO, 1929 and LAUBERT, 1932).

The epidermal cells, however, are not enlarged, though their number is abnormally increased. They keep their healthy appearance until the epidermal layer is burst by the basidia.

In galls which develop on the stems, differentiation of the tissues is also lacking. The anatomical features are similar to those observed in the leaf.

CHAPTER V

THE MATERIAL USED FOR THE EXPERIMENTS

5.1. THE PLANT MATERIAL

Of the Japanese Azaleas grown in Boskoop three cultivars were used, viz. 'Esmeralda', 'Galestin' and 'Moederkensdag', with pink, white and red flowers respectively.

The cultivar 'Hexe' was obtained from Ghent (Belgium). Because of its excellent vegetative growth and flowering, this variety is grown frequently in the Belgian nurseries. An unfavourable characteristic, however, is the susceptibility of this variety to the *Exobasidium* disease.

Specimens of *Vaccinium vitis idaea*, for use in cross inoculation tests were gathered in nature and grown in the garden of the laboratory.

The Azaleas were multiplied by cuttings. Young, recently hardened shoots of about 8 cm length were cut either in April, while the plants were still in the glasshouse, or they were cut in July or August, while growing out of doors. The cuttings were kept in the glasshouse under double glass-cover. The soil was composed of a mixture of 1/3 leaf mold, 1/3 peat moss and 1/3 sand. Roots developed in 4–6 weeks. A treatment with growth substance to stimulate root formation was not necessary. Very favourable conditions for rooting were obtained by the use of clay seedpans and by keeping the humidity high.

The rooted cuttings were potted in the same soil mixture after some stable manure had been added. After some weeks the tips of the shoots were removed, resulting in the unfolding of the axillary buds. Small plants were obtained with 3 to 4 branches which were suitable for the experiments.

5.2. THE ISOLATION AND CULTIVATION OF *Exobasidium japonicum*

Woronin observed the germination of basidiospores of *Exobasidium vaccinii* as early as 1867. Under humid conditions they sprouted in a yeast-like way, forming "conidia". BREFELD (1889) grew the fungus in pure culture in a liquid medium, in which the cells multiplied by sprouting. RICHARDS (1896), however, reported that his attempts to grow the fungus in pure culture, had failed. LOCKHART (1958)

obtained a pure culture of this *Exobasidium* by pouring out a suspension of basidiospores on agar.

MARCHIONATTO (1929) was the first to report the growth of *Exobasidium japonicum* in pure culture. The "conidia" developed on potato-agar. EZUKA (1955) grew *Exobasidium japonicum* from *Rhododendron obtusum* var. *kaempferi* and *Exobasidium vexans* from *Thea sinensis* in pure culture.

GRAAFLAND (1953) succeeded in growing 4 species of *Exobasidium* in pure culture, viz.:

Exobasidium vaccinii Wor. from *Vaccinium vitis idaea*; *Exobasidium rhododendri* Cramer from *Rhododendron ferrugineum* and *Rhododendron hirsutum*; *Exobasidium japonicum* Shir. from *Azalea indica*; *Exobasidium vexans* Massee from *Thea sinensis*.

The following method to grow the fungus in pure culture appeared to be the most suitable one: Young, newly sporulating galls were gathered from the host plants. They were placed in sterile petri dishes, where humidity was kept high by the presence of a plug of wet cotton wool. The galls keep well in this way. If shoots with hypertrophic leaves are used, the galls can be kept fresh for an even longer time by wrapping the cut surface of the shoot in wet cotton wool. The sporulating surfaces of the galls have to be turned as much as possible towards the bottom of the petri dish, in order to allow the forcibly expelled basidiospores to fall on the glass, where they start germinating. Even within 24 hours so many blastospores are formed that they can be taken off with a needle and transferred to a nutrient agar. After 6 to 8 days small, hemi-spherical, glassy colonies are formed with a shiny surface. Gradually they develop the appearance which is typical of *Exobasidium* colonies, i.e. with a sunken centre and peripheral growth. The surface of the mycelial mat gradually shrivels and becomes wrinkled like the surface of a brain. On most of the nutrient media used the colour darkens from yellow to brown. Malt and malt-saleb agar are most favourable for the growth of *Exobasidium* cultures. A temperature of 20–22° C is optimal. The fungi were subcultured every two months.

It is also possible to grow *Exobasidium japonicum*, *Exobasidium vaccinii* and *Exobasidium rhododendri* in nutrient solutions instead of on agar. Shaking is necessary, otherwise the development of the fungus does not proceed beyond the formation of the mycelial mat. With good aeration in a shake culture the fungal particles sprout throughout the solution. This results in a finely divided suspension consisting of cells of irregular shape. A malt solution is most favourable for obtaining such a culture.

Microscopically the length of the hyphae grown on agar varies from 13 to 30 μ, depending on the nutrients used. The width of the hyphae is 1–2 μ. Their shape is irregular, but part of them are fusiform, in which case they are similar to the blastospores formed on the galls of the plants. The particles may also be rectangular or crooked with a knob on one or both ends. Sometimes, especially in old cultures, threads are formed, though the latter can never be

considered to be real hyphae, as the cells are only loosely attached to each other (Fig. 3).

In the nutrient solutions the cells are more uniform; cells cohering in threads are hardly ever found. Though the cells are comparable to the typical *Exobasidium*-blastospores formed on the galls, they are more irregularly shaped. Especially their length is more variable.

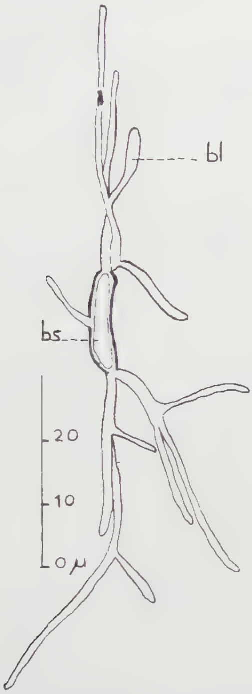

Fig. 3. Germinating basidiospore (bs), forming hyphae and blastospores (bl).

CHAPTER VI

INOCULATION EXPERIMENTS

6.1. Literature

Inoculations with basidiospores taken directly from sporulating galls were performed already by Woronin (1867), who succeeded in infecting healthy plants of *Vaccinium vitis idaea* by means of basidiospores obtained from diseased ones. Richards (1896) obtained a similar result with *Andromeda ligustrina* and *Gaylussaccia resinosa*. Lockhart (1958) observed typical disease symptoms on the young leaves of shoots developing on *Vaccinium myrtilloides* and on *Vaccinium angustifolium*, three months after these plants had been treated with basidiospores. Graafland (1953) inoculated young tea plants with basidiospores of *Exobasidium vexans*, and obtained good results. No information is available with regard to inoculations of Azalea carried out with basidiospores taken from the galls.

Inoculations with blastospores obtained in pure culture were performed by BREFELD (1889). He states, though not very clearly, that the spores of *Exobasidium vaccinii* obtained in pure culture and those formed by sprouting basidiospores have similar effects on *Vaccinium vitis idaea*. GRAAFLAND (1953) obtained good results by inoculating this plant with cells taken from a pure culture of the parasite. Similar results were obtained by LOCKHART (1958) after inoculating *Vaccinium myrtilloides* and *Vaccinium angustifolium* with blastospores of the same fungus. There was no difference between the results obtained with inoculations by means of basidiospores originating from the plants and those obtained with cells grown in pure culture.

6.2. EXPERIMENTS

Well-rooted and vigorously growing cuttings were used for the inoculations. As the galls are already visible when the leaves are a few mm in length it was clear that inoculation had to be performed before the buds began to expand. After the inoculation, however, the dormancy of the buds had to be broken. In order to achieve these two ends axillary buds were inoculated, and the tips of the stems were removed. As, usually, not more than 4 buds per shoot began to expand after the removal of the tip, only the 5 uppermost buds were treated with inoculum. If the plants were too small to be decapitated, the terminal bud was inoculated, although this was more difficult than inoculating an axillary one. The latter consists of a growing point surrounded by only 4 to 6 diminutive leaves, of which 4 are bud-scales. The terminal bud is covered by about 10 of these leaves in different developmental stages. It is not easy to inoculate these tightly packed small organs. Basidiospores and also blastospores, if present, were transferred from diseased plants to healthy ones by means of a moist brush. The inoculated plants were kept for 24 to 30 hours in glass inoculation-chambers, which on the inside were covered with moist filter paper. Then the chambers were opened, and the filter paper was removed. The first symptoms were visible after about 3 weeks, at least if the temperatures had not fallen too much at night. When the nights had been cool, i.e. in September when the temperature could be as low as 0°–5° C, the incubation period was sometimes much longer than 3 weeks.

The number of galls obtained after inoculation was much larger when the plants were decapitated than without this treatment. (Table 1).

TABLE 1

Number of galls on groups of 5 plants inoculated with basidiospores after decapitation, and inoculated without decapitation.

Azalea cultivars	number of galls	
	shoots decapitated	shoots not decapitated
'Esmeralda'	4	0
'Galestin'	14	3

Thus the development of the axillary buds appeared to be favourable for the development of the symptoms. The technique of inoculation was greatly simplified when it was discovered that inoculations could successfully be performed with pure cultures, especially with suspensions from a shake culture. When the culture was 14 days old, the shaking was suspended during one night in order to give the cell material the oppertunity to sink to the bottom of the flask. After discarding the supernatant fluid, the viscose suspension was used as inoculum by brushing it on the buds of the plants. For the first experiment the cultivars 'Esmeralda' and 'Galestin' were used. Half the number of plants were inoculated with basidiospores originating from a gall, and the others were treated with a suspension of the pure culture.

The number of galls which developed on the latter group was even larger than the number that developed on the plants treated with basidiospores (Table 2).

Table 2

Number of galls that developed on groups of 5 plants treated respectively with basidiospores and with suspensions of cells grown in a pure culture.

Azalea cultivars	inoculated with	
	basidiospores	a cell suspension
'Esmeralda'	4	5
'Galestin'	7	9

After such favourable results had been obtained with suspensions, all further experiments were performed with material grown in shake cultures.

Cultures of *Exobasidium japonicum* and *Exobasidium vaccinii* isolated in 1952 have remained virulent up to now, as after inoculation host plants become infected.

CHAPTER VII

THE LIFE CYCLE OF *EXOBASIDIUM* SPECIES

7.1. Literature

The mode of infection of *Exobasidium vaccinii* was revealed by the brilliant observations of Woronin. The basidiospores proved to germinate on the leaves of *Vaccinium vitis idaea*. Sometimes secundary "conidia" were formed by budding, but usually the spores themselves formed germ tubes, which penetrated via the stomata or via the epidermal cells. In the intercellular spaces the branching hyphae developed into a network within 48 hours after the infection.

According to Hilborn & Hyland (1956) the infection of *Vaccinium myrtilloides* and of *Vaccinium angustifolium*, the "lowbush blueberry", by *Exobasidium vaccinii* occurs via the cuticle of the stem. The infection becomes systemic, and the mycelium is perennial in the rhizomes. Much attention has been paid to the way in which the tea plant

becomes infected by *Exobasidium vexans* Massee (Tubbs, 1947; Gadd & Loos, 1948, 1949 and 1950; Reitsma & van Emden, 1949 and 1950; Loos, 1951). This parasite can attack even the 3rd or the 4th leaf reckoned from the tip of the stem, i.e. the leaf which is just fully developed, and also the leaves of a developing bud. The basidiospores of this parasite do not form blastospores, but they develop a germ tube. From an appressorium on the cuticle a hypha may penetrate the outer epidermal cell wall.

Only little is known with regard to the way in which Azaleas are infected by *Exobasidium japonicum*. According to Marchal (1925) and Marchionatto (1929) the germ tubes penetrate into the young leaves during the unfolding of the buds.

Eftimiu & Kharbush (1927) studied the cytology of *Exobasidium* species extensively. They studied the spores, the mycelium, and the basidia of an *Exobasidium* occurring on Azaleas grown in the vicinity of Paris. Though these authors considered this fungus to be *Exobasidium discoideum* Ellis, it can be concluded, especially from their illustrations, that it was *Exobasidium japonicum* Shir. According to these authors, the basidiospores of this fungus are monocaryotic, whereas the cells of the intercellular mycelium are dicaryotic. Also in the young basidia two distinct nuclei are present, which fuse to a diploid one. After two divisions the latter gives rise to the 4 haploid nuclei of the 4 basidiospores.

7.2. Experiments

It was not yet known in what stage of the life cycle the diploidization occurs, of which the result is recognizable in the dicaryotic condition of the mycelium found in the leaves. To answer this question we had to know first of all whether the basidiospores, and also the blastospores really are uninucleate. Secondly, the possibility of a cell fusion outside the host plant had to be investigated. For that purpose basidiospores from a mature gall caught on a glass slide were allowed to germinate, after which the nuclei were stained with haematoxylin. The basidiospores, as well as the blastospores developed out of the former, appeared to be uninucleate (Fig. 4 and 5). The possibility of a fusion between these spores was studied in a moist chamber culture. A small piece of a mature gall was glued to the bottom of the chamber inside the glass ring, with the sporulating side directed towards the cover slip; on the latter a drop of malt agar had been placed (5 % malt in 1 % agar). By keeping the moist chamber upside down for several hours, we obtained that the basidiospores collected on the agar. Nearly every spore germinated, which could be observed microscopically. The contents of the spores moved towards the 2 poles, after which germination of the spores took place on either side, resulting in the formation either of 2 blastospores or of 2 hyphae each bearing a terminal blastospore. In the meantime the spore itself became divided by one or more septae. No sign of cell fusion was ever observed: neither were basidiospores lying in the neighbourhood of each other, nor blastospores originating from different basidiospores

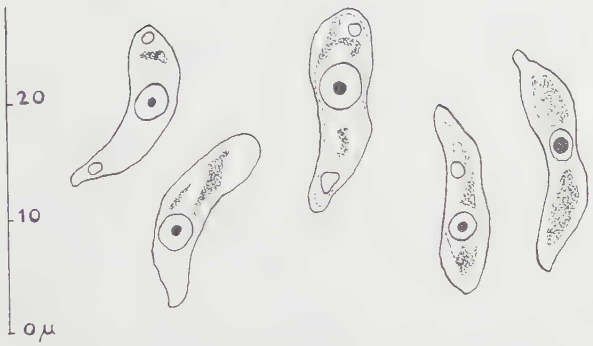

Fig. 4. Basidiospores with nuclei stained with haematoxylin.

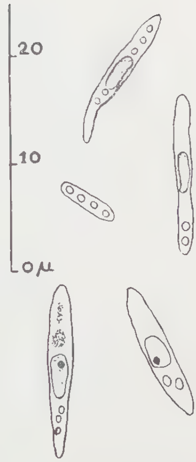

Fig. 5

Fig. 5. Blastospores developed in a shake culture, stained with haematoxylin.

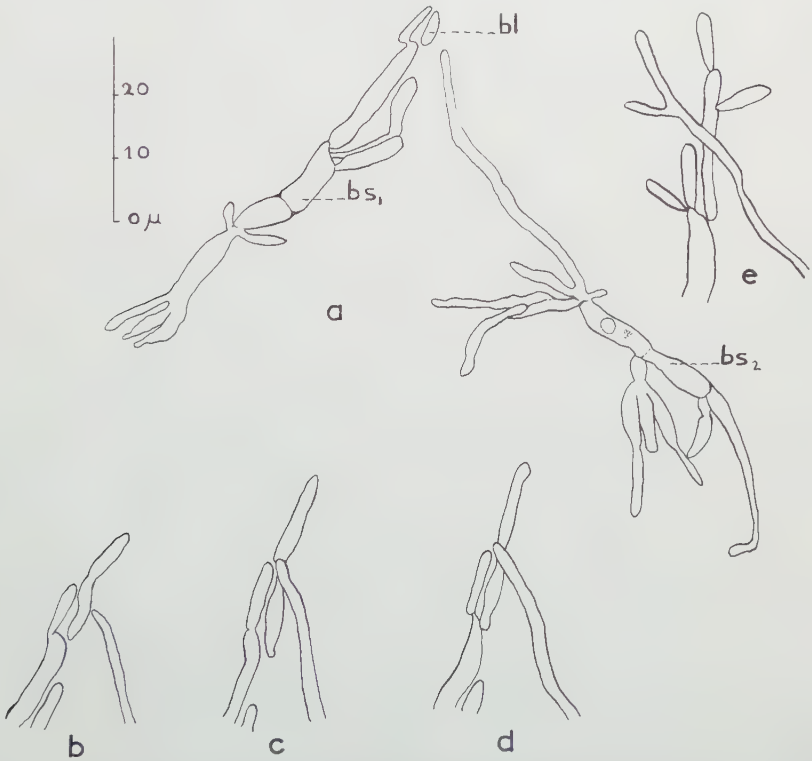

Fig. 6. *a*: Two germinating basidiospores (bs_1 and bs_2) originated from one basidium, forming hyphae and blastospores (bl). *b*, *c*, *d* and *e*: Blastospores and tips of hyphae of *a*, after 5, 10, 29 and 45 hours, respectively.

ever seen conjugating even though they were separated from each other only by a small distance. The colonies of blastospores grew against each other without making contact (Fig. 6). In many moist chamber cultures the basidiospores were found in groups of 4 or 5, probably originating from the same basidium. If + and — spores existed, it might be expected that both would be represented in these groups. Therefore, special attention was paid to these cases, but even here no fusion was observed. It may be concluded that in vitro all spores behave in a similar way.

Another possibility that had to be investigated, is that the dicaryotic mycelium in the young leaves develops after a union of the spores or of the germ tubes either on the surface of the leaves or after penetration. If the fungus were heterothallic, at least one + and one — spore would be required to accomplish an infection. In case the fungus were homothallic, only one spore would be necessary for the development of a gall. To investigate this question it was necessary to dispose of monospore cultures.

Monospore cultures were obtained from a suspension of a shake culture by diluting the latter so far that a drop taken on a needle contained only one cell. Such a drop was placed on the malt agar sticking to the cover slip of a moist chamber culture. After it had been ascertained by microscopic examination that only one spore was present, the latter was allowed to develop into a colony. After one to two weeks the colony was transferred to malt agar. In this way about 10 monospore cultures were obtained. Each one was tested on a group of 5 test plants as to its ability to cause infection. Five of these cultures proved to cause infection on all 5 plants, 2 of them caused infection on 4 of the group of 5 plants and 3 monospore cultures caused galls on 3 out of a group of 5 plants. Thus it may be concluded that the fungus is homothallic, because sporidial cells derived from a single blastospore are able to develop into a dicaryotic mycelium.

Monobasidiospore cultures were obtained in moist chamber cultures as described before (p. 360). The time during which the chamber had to be kept upside down, however, was now but short. A period of one to two minutes appeared to be most suitable to obtain basidiospores separated from each other by such a distance that the colonies into which they developed did not touch. This was controlled microscopically. If more than 10 basidiospores were present on the cover slip, the culture was discarded. In many cultures only one basidiospore was present, in others 2 to 5. In three cases it was possible to isolate the spores of such a group and to raise separate colonies from them. All in all 34 monobasidiospore cultures were obtained, among which 21 originated from basidiospores belonging to different basidia. In two instances it was possible to grow a colony out of each of 4 basidiospores which probably originated from the same basidium. Once, 5 colonies were obtained from 5 basidiospores which were probably derived from the same basidium. These cultures were tested separately on Azalea plants. They all caused infection (Table 3).

TABLE 3

Number of plants developing galls after inoculation with monobasidiospore cultures

number of monospore cultures; each one was used to inoculate 5 plants	numerator: number of plants with galls / denominator: number of plants inoculated
9	45/9 × 5
6	24/6 × 5
6	18/6 × 5
2 } basidiospores originating from one basidium	10/2 × 5
1	4/1 × 5
1	3/1 × 5
1 } the same	5/1 × 5
3	12/3 × 5
2 } the same	10/2 × 5
1	4/1 × 5
2	6/2 × 5

These experiments prove that *Exobasidium japonicum* is homothallic. Monocaryotic cells, formed by a process of budding out of one basidiospore and, therefore, genotypically identical, penetrate the outer wall of the epidermis of the young leaves. In the leaf tissue the dicaryotic mycelium, therefore, is formed out of cells between which there can be no difference in sex.

CHAPTER VIII

CROSS INOCULATIONS

8.1. CROSS INOCULATIONS WITH *Exobasidium* SPORES ORIGINATING FROM DIFFERENT AZALEA CULTIVARS

The *Exobasidium* galls are found on the Japanese *Azalea obtusum* varieties grown in Boskoop as well as on the cultivars grown in Ghent, which have *Rhododendron simsii* as ancestor. The latter seem to be very susceptible to the disease. It is as yet uncertain whether these galls are caused by two different parasites, each occurring on a different group of cultivars or by only one parasite, causing galls in all cultivars. Morphologically no differences could be found between the shape of the galls on the different cultivars, and the basidiospores too proved to be identical, measuring 13–18 × 2.5–5.5 μ. However, physiological specialization might occur. To study this question, cross inoculations were performed with basidiospores as well as with cell suspensions from shake cultures, the one like the other originating from infected plants of the Japanese cultivars 'Esmeralda' and 'Galestin', and from infected plants of the Belgian cultivar 'Hexe'. Table 4 shows that the parasite from the Japanese Azalea could cause galls on the Belgian cultivar and vice versa. The Japanese cultivars could be infected with the fungus from 'Esmeralda'. The parasite from 'Hexe' easily caused infections on plants of the same cultivar, but

Table 4

Number of galls developing on different Azalea cultivars after inoculation with *Exobasidium* of different origin.

Azalea cultivar	origin of the *Exobasidium* inoculum	number of galls / number of plants [1]	number of diseased plants
'Esmeralda'	'Hexe'	16/6	
'Galestin'	'Hexe'	25/6	
'Esmeralda'	'Esmeralda'	37/6	
'Galestin'	'Esmeralda'	36/6	
'Esmeralda'	control	0/6	
'Galestin'	control	0/6	
'Hexe'	'Hexe'	76/20	19
'Esmeralda'	'Hexe'	46/20	17
'Hexe'	'Esmeralda'	42/20	19
'Esmeralda'	'Esmeralda'	31/20	12
'Hexe'	control	0/20	0
'Esmeralda'	control	0/20	0

[1]) numerator: total number of galls; denominator: number of inoculated plants.

was less apt to provoke galls on the Japanese cultivars. Conversely, the fungus from the latter was able to cause infections on 'Hexe', even to a higher degree than on the cultivars from which it originated.

Though some specialization seems to occur, *Exobasidium japonicum* is certainly not narrowly specialized on certain Azalea varieties, as the parasite may attack Azaleas of different genetic composition.

8.2. Cross inoculations with *Exobasidium* spores originating from different less closely related host plants

According to Savile (1959) and other authors, the *Exobasidium* species occurring on Azalea are identical with *Exobasidium vaccinii* described by Woronin. This opinion is based on the morphological similarity of these fungi. However, even if they are considered to be a single species, this does not mean that the *Exobasidium* strains occurring on different host plants might not be specialized in their pathogenicity. If they are different physiological races or biotypes they may be expected to occur on different host plants. To investigate the parasitic character of some *Exobasidium* isolates from different origin, cross inoculations were performed with pure cultures obtained from the Belgian Azalea cultivar 'Hexe', from the Dutch cultivar 'Esmeralda' and from *Vaccinium vitis idaea*. Graafland (1953) previously succeeded in obtaining infections of the latter by inoculating it with a pure culture of *Exobasidium vaccinii.*

Cross inoculations in the experiments described in this paper were performed with pure cultures of *Exobasidium japonicum* obtained from different Azalea cultivars and of *Exobasidium vaccinii* obtained from *Vaccinium vitis idaea.*

Pure cultures of both fungi on agar showed a different aspect. The brown colonies of the former produced a dark discolouration

of the nutrient agar. The light yellow colonies of the latter hardly changed the colour of the agar. Both parasites also showed a difference in colour of the nutrient solution when grown in shake cultures.

From cross inoculations it became clear that *Vaccinium vitis idaea* was not attacked by the *Exobasidium* from Azalea, and that conversely Azalea does not function as a host plant to the fungus living on *Vaccinium*. In all 6 sets of experiments were made, all with similar results. The results of one of these sets of experiments are given in table 5.

TABLE 5

Number of galls resulting from inoculation of Azalea and *Vaccinium vitis idaea* plants with pure cultures of *Exobasidium* of different origin; 20 test plants in each set.

origin of the pure culture	test plants	number of galls	number of diseased plants
	Azalea cultivars:		
Exobasidium japonicum	'Esmeralda'	25	17
,, ,,	'Hexe'	40	19
,, ,,	*Vaccinium vitis idaea*	0	0
	Azalea cultivars:		
Exobasidium vaccinii	'Esmeralda'	0	0
,, ,,	'Hexe'	0	0
,, ,,	*Vaccinium vitis idaea*	22	15

Despite their morphological similarity, there is a difference in pathogenicity between these fungi. It is possible, therefore, to consider them to represent physiological races of one species, viz. *Exobasidium vaccinii* Wor. If, however, the physiological specialization of the fungus and the difference in aspect of the pure cultures are considered to be of sufficient importance to distinguish more species, then the valid name for the fungus on Azalea would be *Exobasidium japonicum* Shir. This standpoint is taken up in the present paper, at least if we assume that the American species *Exobasidium azaleae* Peck and *Exobasidium discoideum* Ellis are different too.

It is regrettable that the set of cross inoculations carried out with *Exobasidium* cultures originating from Azalea and from Swiss *Rhododendron* species remained incomplete, as specimens of the latter were not available. *Exobasidium rhododendri*, parasite of the Swiss *Rhododendron ferrugineum* was obtained in pure culture (GRAAFLAND, 1953). On malt agar this fungus differs from *Exobasidium vaccinii* Wor. and *Exobasidium japonicum* Shir. in developing colonies with a more finely wrinkled surface and with a grey-brown rather than a yellow or brown colour. 'Hexe' was inoculated with this pure culture, but no galls appeared. Though there was no opportunity to inoculate *Rhododendron ferrugineum* with *Exobasidium* from 'Hexe', and the evidence, therefore, is not complete, it may be assumed that the fungus from this *Rhododendron* must also be considered as a physiologically specialized form.

CHAPTER IX

DEVELOPMENTAL STAGES OF THE BUD AT WHICH INFECTION WITH *EXOBASIDIUM JAPONICUM* IS POSSIBLE

Efforts were made to obtain more information concerning the developmental stages of the young leaves which are sensitive to infection by this fungus. For that purpose dormant axillary buds of Azalea shoots were inoculated with an *Exobasidium* suspension immediately after the shoots had been decapitated and the buds had started to develop. At different intervals after inoculation the axillary buds were removed and fixed in Craf's solution. In this way the developmental stages of the young leaves and the progress of the gall formation could be studied 0, 1, 2, 3, 4, 5, 7, 10, 14, 21 and 28 days after the inoculation.

Microtome slides revealed that within 4 days after the inoculation the infected young leaves showed hypertrophy and hyperplasy. All differentiation between the palissade layer and the spongy parenchyma proved to be lacking. In a few cases hyphae were visible in the intercellular spaces, though the process of penetration itself was not observed. The small size of the blastospores made it difficult to detect them between the hairs and the scales which cover the epidermis of the young leaves. However, it became clear that the young leaves are already sensitive to infection when they are still enclosed in the bud.

The aim of the following experiment was to study the length of the period during which the young leaves could still be infected. For

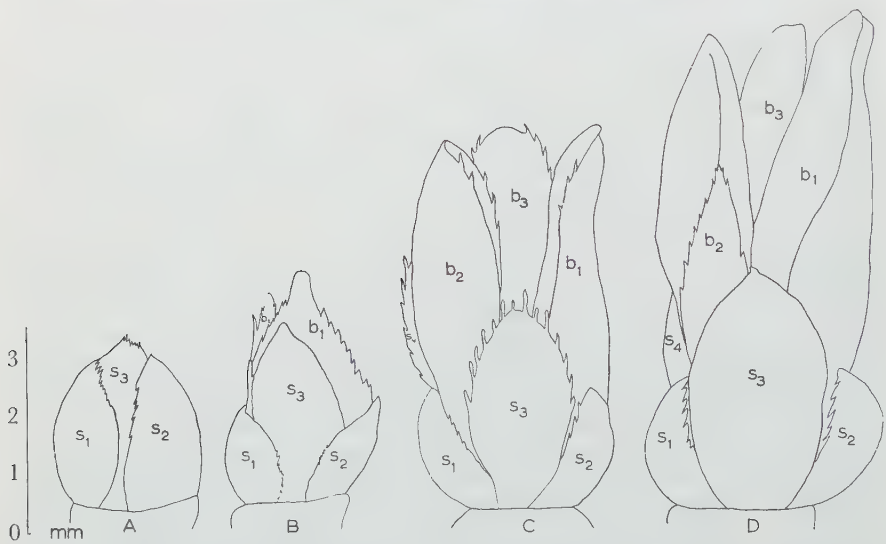

Fig. 7. A–D: development of an axillary bud of *Azalea indica*, cultivar 'Hexe'. b_1-b_3: leaves. s_1-s_4: bud scales.

that purpose in 70 Azalea plants the tips of the shoots were removed in order to stimulate the unfolding of the buds. At different intervals after this unfolding, the buds were inoculated with a pure culture of *Exobasidium*. Ten plants were used for each set of inoculations. It was found that leaves of 8–10 mm length still could be infected. In the next stage of development the laminae begin to spread out, and in that stage the leaves are no longer susceptible to infection (Table 6; Fig. 7 and Plate 1, C–G).

Table 6

Number of galls on the leaves of axillary buds inoculated in different developmental stages i.e. at different moments after the decapitation of the shoots.

number of days after decapitation	length of the axillary buds in mm	number of galls per group of 10 plants	number of plants with galls
0	1.5	70	10
11	2.5	85	10
19	3.0	30	9
25	4.0	17	8
30	6.5	42	10
40	8–10	1	1
50	12–15	0	0

The stage of development at which a leaf is still subject to infection, was studied also in another way. The young just visible leaves of 40 plants were inoculated with a pure culture of *Exobasidium*; the 4th visible leaf reckoned from the tip of the shoot being the last to be inoculated. The inoculated leaves were marked with paint, at least when they were not too small for this treatment. After 5 weeks 42 galls had appeared on those leaves which had developed after the inoculation. Twelve galls were present on those leaves which at the time of inoculation were about 8 mm in length and just large enough to be marked. This result is in agreement with that of the former experiment: the leaves are only susceptible to infection in a juvenile stage. As soon as the laminae begin to spread out, infection is no longer possible.

From a practical point of view it was especially important to answer the question how long inoculum of *Exobasidium* remains viable on or in dormant buds. In the greenhouses the Azaleas may already be infected by the spores of *Exobasidium* galls long before the growers start cutting or pruning, and, therefore, long before the dormancy of the axillary buds is broken. An experiment was performed with artificially administered inoculum. Nine groups, each of 5 plants, were inoculated with a pure culture. In each plant the youngest visible leaf was marked with paint. On every shoot the 4 axillary buds inserted below the marked leaf were inoculated. The plants were then kept at high humidity for 30 hours. At different intervals, up to 4 weeks after the inoculation, the tips of the shoots were removed just above the marked leaf to stimulate the development of the buds. It appeared that even buds inoculated 4 weeks before they began to

expand, showed gall development (Table 7, experiment 1). The experiment was repeated in the same way, but instead of removing the tips of the shoots within a period of at the most 4 weeks after the inoculation, longer intervals were chosen. Even 6 months after the inoculation of the dormant buds the fungus still appeared to be viable, for galls were found in buds that remained dormant for half a year after they had been treated with the suspension (Table 7, experiment 2).

Table 7

Number of galls occurring on young leaves developing at different intervals after inoculation with an *Exobasidium* suspension; five plants in each group.

	Number of days after the inoculated buds begin to expand	number of galls	number of plants with galls
experiment 1	0	17	5
	2	20	5
	3	18	5
	5	16	5
	9	10	4
	12	7	5
	18	3	3
	23	4	3
	30	4	3
experiment 2	14	14	4
	24	11	5
	28	5	3
	35	11	4
	56	2	2
	91	3	3
	119	2	2
	182	4	2

These experiments shed no light on the question how the fungus behaves during the period between the inoculation and the development of the gall, and how it obtains the necessary nutrients. It is possible that the blastospores administered to the buds start budding on their surface, and that the colonies thus formed remain viable between the hairs and the scales of the dormant buds. Penetration of the young leaves may occur at the time the buds begin to expand. However, it is also possible that the spores penetrate directly into the leaves of the dormant bud, and that the development of the hyphae inside the latter is delayed as long as the buds remain dormant. Which of these two pathways actually is taken, remains undecided as the first developmental stages of the disease could not be detected. The rate of development of the host plant determines the time at which the symptoms of the disease will appear. If infected axillary buds are stimulated to new growth, a parallel development of host and parasite takes place. Thus the incubation period depends on the environmental conditions: if the buds are just unfolding at the time

of the contamination, the symptoms may appear within twenty days after the infection but they may also be delayed for 6 months or longer after the infection, if the buds remain dormant for that period.

CHAPTER X

THE CONTROL OF THE DISEASE

10.1. Hygienic measures

In the literature the importance of removing and burning the galls has frequently been pointed out (Naumann, 1909; Miles, 1928; White, 1933; Stautemas, 1951; Jaenichen, 1954). This is sometimes done in combination with spraying with Bordeaux mixture. The "Tuinbouwgids" of 1960 (Dutch Horticultural Guide) still recommends these measures, though it is known that they are not conclusive (Welvaert, 1952; Graafland, 1957). This method would be satisfactory if the galls were removed in time, i.e. before sporulation, when the colour is still green, but the picking of mature galls promotes the spread of the basidiospores. However, it is impractical to remove all gall bearing leaves sufficiently early from large numbers of densely planted Azaleas. Moreover, before they begin to expand, infected buds cannot be distinguished from healthy ones. Thus a continual control of the unfolding buds would be necessary. Even if the fungus were eradicated in this way from a greenhouse, a new contamination could easily occur from spores blown in from neighbouring places.

It is obvious that after removing the galls only once, the plants may still be diseased. Plants imported from Belgium in an apparantly healthy state may show galls at the time the dormant buds are unfolding. For that reason *Exobasidium japonicum* may easily be introduced into other countries, because it may be hidden in dormant buds of imported Azalea plants. In this manner it is easy to defeat the quarantine laws, which list the fungus as a dangerous parasite and forbid its importation.

10.2 Control by fungicides

In the literature spraying with fungicides has frequently been advised. Marchal (1925) recommends the use of lime after pruning; Miles (1928) and Marchionatto (1929) prefer Bordeaux mixture, though Marchionatto also mentions lime. Cole (1944) and Thiel (1951) also consider Bordeaux mixture to be an effective spray; Thiel recommends the use of lime sulphur. Ciferri (1953) mentions zincethylenebisdithiocarbamate as a good preventive fungicide. Jaenichen (1954) prefers a treatment with carbamates following the removal of the galls. Anonymus (1956) had good results by spraying with copperoxychloride or zineb after the galls had been removed as far as possible.

Many authors consider insects to be effective in transmitting spores from mature galls to other parts of the plants. For that reason the control of insects is important (Stautemas, 1951). Next to insects

mites might be involved, and these animals too should, therefore, be killed (MILES, 1928; MARCHIONATTO, 1929). Marchionatto based this opinion on his observation of basidiospores attached to the bodies and the mouth parts of these arthropods. MARCHAL (1925) regards *Aleurodes vaporarius* as causing the spread of the spores. Though it is possible that insects and mites play a rôle in the transfer of *Exobasidium* spores from galls to healthy parts of the plants, the disease is in our opinion mainly spread by water droplets carried by draughts arising when the glasshouses are aired and augmented by spray irrigation. In whatever way contamination occurs, protection of the buds with a fungicide is necessary. Fungicides have to be applied regularly, and not alone before or after pruning. Some growers even spray every fortnight with copper compounds, and succeed in this way in keeping their plants free from the *Exobasidium* disease as well as from other parasitic fungi, such as *Septoria azaleae* Vogl. A drawback is that the plants are more or less severely damaged by such frequent sprayings. They shed their leaves, and become unattractive for sale. Spraying during the period of flowering is disastrous.

10.3 EXPERIMENTS WITH FUNGICIDES IN VITRO

In the "Tuinbouwgids" of 1960 (Dutch Horticultural Guide) copper compounds or lime sulphur are advised for spraying. As these fungicides are more and more going out of use, some of the newer compounds, i.e. zineb and captan, were assayed against the *Exobasidium* disease.

The irregularly shaped blastospores developing in a shake culture can be obtained in large numbers, but they can hardly be used in the ordinary germination tests in vitro, in which the number of germinating spores is counted, as only a small percentage are budding. It is also impossible to assess the E.D. 50 of the basidiospores, as they do not germinate at all in watery suspensions. Budding only occurs when the spores are covered with a thin film of condensation-water, i.e. when kept at a high humidity. Even if basidiospores could be used in assaying, hundreds of Azaleas would have to be inoculated in order to obtain the high numbers of spores needed for the experiments. To avoid these difficulties, different concentrations of the fungicides were added to the nutrient solution of shake cultures. The inhibition of the growth of the blastospores could be determined by weighing the mycelium. The following fungicides were used in concentrations varying from 1/10 to 1/100 of those used in practice:

copperoxychloride:	0.05,	0.10,	0.20 and 0.50	g/l
lime sulphur:	0.075,	0.15,	0.30 and 0.75	cc/l
zineb [1])	0.025,	0.05,	0.10 and 0.25	g/l
captan [2])	0.02,	0.04,	0.10 and 0.20	g/l

The experiments were carried out in erlenmeyer flasks containing

[1]) zincethylenebisdithiocarbamate.
[2]) 4, 5– cyclohexene –1, 2– dicarboximide.

50 cc of a malt solution to which known quantities of water and of a standard solution had been added. The media containing copper-oxychloride were prepared as indicated in table 8.

TABLE 8

Composition of the media containing copperoxychloride.

malt solution in cc	water in cc	copperoxychloride quantity used of a 0.1 % standard solution in cc	copperoxychloride concentration in g per l
50	45	5	0.05
50	40	10	0.1
50	30	20	0.2
50	0	50	0.5

The other media were prepared in a similar way. Thus solutions were obtained equal in nutritional value to the solutions that are normally used. To all erlenmeyer flasks 1 cc of an *Exobasidium japonicum* shake culture was added. Similar suspensions without fungicides as well as malt solutions without any addition were used as controls. After all cultures had been shaken for $2^1/_2$ to 3 weeks, the quantity of mycelium which had developed, was determined. The test cultures were filtered through a Buchner filter covered with filter paper. The latter with the mycelium was dried at a temperature of 105° C–110° C for 3 to 7 days, and then weighed. The experiments were replicated three times. It turned out that copperoxychloride suppressed the growth of the mycelium if used at the highest concentration (0.5 g/l). Lime sulphur also inhibited the growth of the mycelium, even in concentrations of 0.15 cc/l. Zineb did not seem to be of any influence; this fungicide even stimulated the growth. Though captan inhibited the growth to a high degree in concentrations of 0.2 g/l, this fungicide was of no influence at a concentration of 0.1 g/l. It was concluded that the effect of this fungicide and that of lime sulphur were most favourable (Fig. 8). Since the latter is now less widely used in practice, captan was chosen for the experiments with test plants.

10.4 EXPERIMENTS WITH FUNGICIDES IN VIVO

Captan was used in concentrations of 2 g/l and 1 g/l. The buds of Azalea shoots were treated with the fungicide before, on the same day, and after the day on which they were inoculated with a pure culture of *Exobasidium*. In one series of experiments the tips of the shoots were removed simultaneously with the application of the fungal suspension. In a second series the tips were removed 10 days after the inoculation. It was inadvisable to remove the tips a long time before treating the plants with captan, as in that case the unfolding buds would develop their infections before the captan could have exercised its fungicidal action. In each series 4 plants were used with a total of 36 buds. From 4 control plants the tips of the shoots were

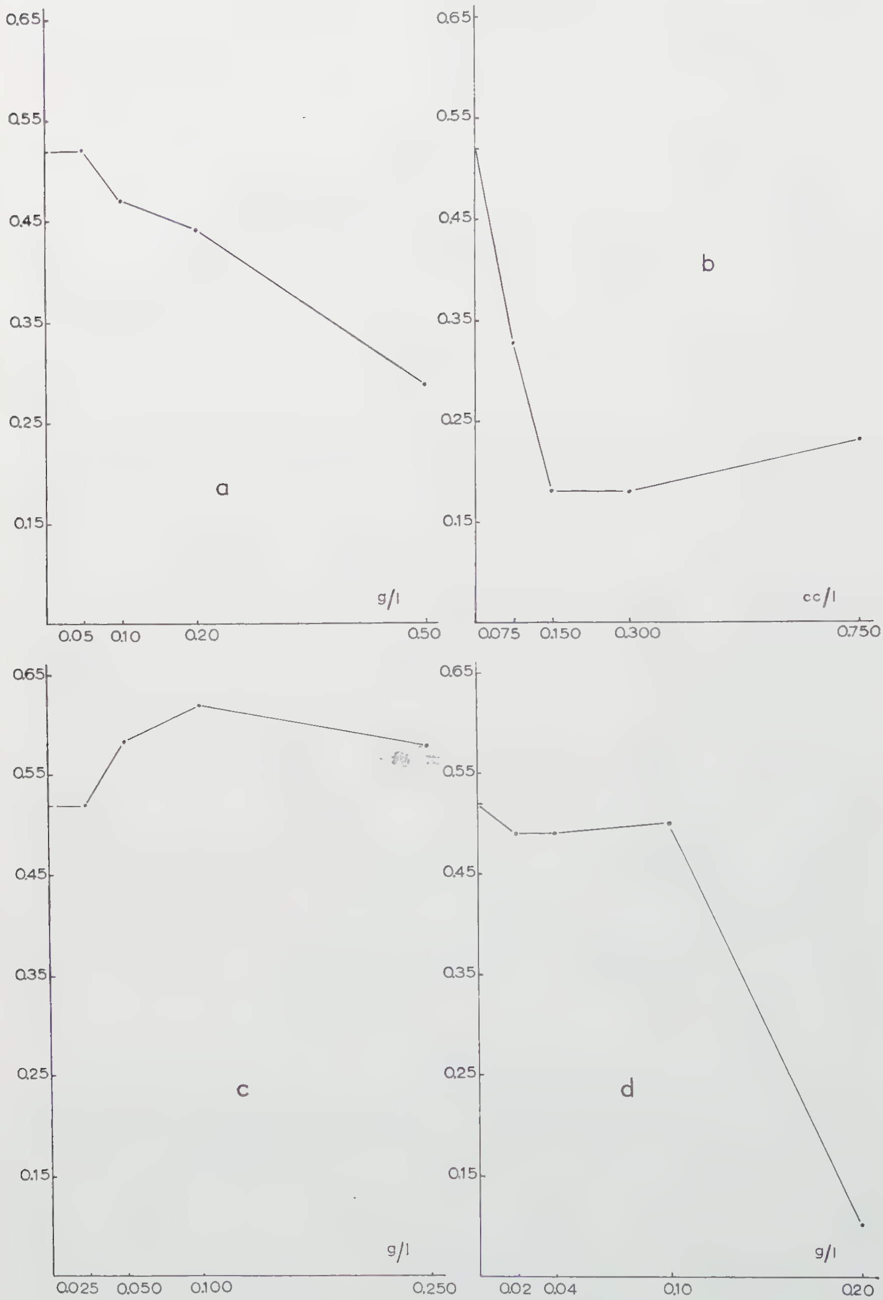

Fig. 8. Influence of fungicides on the dry-weight of mycelium formed by *Exobasidium japonicum* in shake cultures, *a*: copperoxychloride, *b*: lime sulphur, *c*: zineb, *d:* captan. Dry-weight of the mycelium without fungicides 0.52 g. Abscis: concentration in g or cc per l; ordinate: weight in g.

removed simultaneously with the inoculation, and from 4 other control plants this was done 10 days after the inoculation.

The smallest number of galls appeared when a captan suspension of 2 g/l was applied simultaneously with the fungal suspension (Table 9). The concentration of 2 g/l is the one that is also used in practice.

Table 9

Number of galls developing on groups of 4 Azalea plants with 36 buds in total, after inoculation with a suspension of *Exobasidium japonicum* and treatment with captan.

time of treatment with captan in relation to inoculation time	tips of the shoots removed simultaneously with inoculation		tips of the shoots removed 10 days after inoculation	
	concentration of captan		concentration of captan	
	1 g/l	2 g/l	1 g/l	2 g/l
2 days before	42	14	29	19
simultaneously	8	3	1	0
1 day later	20	15	8	6
7 days later	23	13	14	9

controls: 4 plants with the tips removed simultaneously with the inoculation: 34 galls
4 plants with the tips removed 10 days after inoculation: 24 galls.

From these experiments it became clear that, in case the buds remained dormant, the fungus was killed by captan when the latter was added simultaneously with the inoculum. If captan was administered one day or one week after the inoculum, galls became visible when the infected buds began to unfold. Perhaps the captan could not reach the sprouting blastospores because the latter were hidden between the scales of the buds, or because the fungus had already penetrated into the young leaves before the captan was applied.

In addition some minor experiments were performed with the other fungicides that had been tested in vitro. In these experiments the following solutions were used:

copperoxychloride 5 g/l
zineb 2.5 g/l
lime sulphur 7.5 cc/l

The test plants were sprayed one week after the inoculation, and the shoots were decapitated 10 days after that treatment. Each series was composed of 4 plants with in total about 30 axillary buds. The result is indicated in table 10.

The main series of experiments showed that fungicides are able to keep the fungus under control, provided that they are administered at the time of infection. This means that the buds should continuously be protected, as danger of infection is always present, particularly in those glasshouses where sporulating galls occur frequently.

Instead of spraying frequently, the application of hygienic measures seems to be more recommendable. Discarding the young galls before

TABLE 10

Number of galls developing on groups of 4 Azalea plants with 30 axillary buds in total after inoculation with a suspension of *Exobasidium japonicum* and treatment with fungicides.

fungicides	number of galls
copperoxychloride	3
zineb	8
lime sulphur	11
control plants	24

they sporulate, destroys the source of infection. This practice has also been recommended in the "Tuinbouwgids" (Dutch Horticultural Guide). However, it is difficult to pick out all young galls at the proper time. Therefore, in addition the grower may apply a fungicide to prevent infection, especially when sporulating galls are found. Spraying is also important when vegetative growth is resumed after pruning or after cuttings have been taken.

CHAPTER XI

DISCUSSION ON THE RELATIONS BETWEEN THE GENERA *EXOBASIDIUM*, *USTILAGO*, *TILLETIA* AND *TAPHRINA*

According to GÄUMANN (1949), the genera *Exobasidium* and *Kordyana* compose the family *Exobasidiaceae*. ALEXOPOULOS (1952) considers this family to be one of seven belonging to the order *Agaricales*, which according to others is synonymous with the order *Hymenomycetes* of the subclass *Holobasidiomycetes*, class *Basidiomycetes*. The *Exobasidiaceae* are primitive forms, lacking basidiocarps, and assignable to the *Agaricales* only on account of the presence of a hymenium consisting of basidia; this hymenium is developed just below the surface of the host plant and becomes exposed by the bursting of the cuticle. Because of this primitive character some authors, e.g. VIENNOT–BOURGIN (1949), assign the genera *Exobasidium* and *Kordyana* to a separate order: the *Exobasidiales*. Others, e.g. TALBOT (1954), are of opinion that the genus *Exobasidium* must be assigned to the subclass *Hemibasidiomycetes*, as there is a resemblance to the *Ustilaginales*. This resemblance is found in the fact that the *Exobasidiaceae* are parasites, and also in the budding of their basidiospores resulting in the formation of colonies; this reminds one of the budding of the sporidia arising from the smut spores of *Ustilago* species, such as *Ustilago hordei* (Pers.) Lagerh. Moreover, there is a striking resemblance between the characters shown by *Exobasidium* and by *Ustilago* species when grown in pure culture on an agar medium. Both fungi develop colonies with a wrinkled or folded surface.

In *Ustilago* species the process of budding is followed by conjugation of + and — sporidia. In the genus *Tilletia*, e.g. in *Tilletia caries* (D.C.) Tul., conjugation occurs between the sporidia as soon as they are formed. In this way in these two genera a dicaryotic

mycelium is formed. In the genus *Exobasidium*, however, conjugation has never been observed, though it is known that a dicaryotic mycelium is present in the host plant. This lack of conjugation is another point of difference between *Exobasidium* and the *Ustilaginales*. Though the genera *Exobasidium* and *Taphrina* are not closely related from a taxonomic point of view, the life cycles of both fungi show a striking ecological resemblance. This has already been pointed out by WORONIN (1867), who mentions the following important similarities:

1. Both fungi are true parasites. They cause galls of a similar shape or, eventually, witches' brooms.
2. In both cases the mycelium lives in the intercellular spaces of the tissues. A layer of hyphae develops either between the epidermis and the cuticle of the galls or underneath the epidermis. The cuticle is burst at the time of sporulation.
3. In both cases germination of the spores occurs by budding.

Considering these similarities it is not astonishing that some authors consider both genera to belong to one group of which the members show the same kind of parasitism (THOMAS, 1897; JUEL, 1912). LAUBERT (1932) too mentions the analogy between the representatives of the genera *Taphrina* and *Exobasidium* in their way of living and in their influence on the host plants. In both genera fungi occur which cause galls and also witches' brooms. The galls show the same anatomical picture: a uniform, coarse parenchyma with few intercellular spaces and a scarcity of chloroplasts. In both cases the hyphae are thin, 0.5–1.0 μ in width, and grow in the intercellular spaces. The infection occurs by monocaryotic, haploid spores; the mycelium is dicaryotic. In *Exobasidium japonicum* no copulation of spores was found. In the genus *Taphrina*, according to MIX (1949), copulation seems to be exceptional. Only *Taphrina epiphylla* Sadeb. and *Taphrina deformans* (Berk.) Tul. show copulation of ascospores or of blastospores derived from the ascospores (WIEBEN, 1927; MIX, 1935). In the latter species, however, conjugation is rare. The difference between the genera exists in the presence of basidia in the genus *Exobasidium* and of asci in the genus *Taphrina*. The species of the former genus are mainly parasites on representatives of the *Ericaceae* in contrast to those of *Taphrina*, which do not occur on members of this family.

The method used by GRAAFLAND (1953) in cultivating *Exobasidium* species was originally used by MIX (1949) for growing *Taphrina* species. The cultures are in both cases alike, consisting of short hyphae, budding like yeast cells. The length of these short hyphae is not constant, but depends on the composition of the nutrient solution. MIX (1949) used the name "blastospores" for these yeast-like hyphae. He agrees with FITZPATRICK (1934) that these spores are infectious. Thus, the development, the multiplication by budding, the shape and the infectious character are similar in *Taphrina* and in *Exobasidium*. Mix's term "blastospores" can thus be taken over for *Exobasidium*. On the other hand WORONIN (1867) called the sprouting cells "conidia". It seems, however, preferable to restrict this name to spores borne on conidiophores, as it is customary nowadays.

According to AINSWORTH and BISBY (1950), the term "sporidia" should be confined to the secundary spores formed by the *Ustilaginales* and *Uredinales*, where the process of budding is followed by conjugation of the sporidia.

Therefore, to avoid all confusion, in this paper the term "blastospores" has been used in accordance with the definition given by Ainsworth and Bisby: "a spore which has been budded off".

When comparing the parasitic character of representatives of the genera *Exobasidium* and *Taphrina*, it must be concluded that both genera are biologically very similar. This is remarkable, as the genera differ widely from a taxonomic point of view. In *Exobasidium* the spores are exogenous, borne on a basidium; in *Taphrina* they are endogenous, formed in asci. However, here the difference stops, for the behaviour of these spores is similar. By sprouting both may form blastospores, which may sprout once more. The basidiospores of *Exobasidium*, the ascospores of *Taphrina*, as well as the blastospores that are formed in both genera, are able to infect their host plants. One can imagine that the fungi belonging to these genera are of the same origin, and that, by differentiation, one group with endogenous and another group with exogenous spore formation have developed. However, it is also possible that the similarity in behaviour between these groups, so different in the taxonomically important mode of development of the spores, has been induced by their parasitic way of living. [1]

SUMMARY

1. The purpose of this investigation was to study the life cycle of *Exobasidium japonicum* Shir., a fungus parasitic on Azalea. The relation of this parasite to its host plant and the mode of infection were studied, as well as some control measures important to commercial growers. Attention was also paid to the question of the identity of this fungus with that described by Woronin as *Exobasidium vaccinii*, parasitic on *Vaccinium vitis idaea.*

2. For this study cultivars of the genus *Rhododendron* were chosen as host plants. Cultivars of Japanese origin viz. 'Esmeralda', 'Galestin' and 'Moederkensdag', frequently grown in Boskoop, and the cultivar 'Hexe', mainly grown in Ghent (Belgium), were used. Azaleas are cultivated during the greater part of the year in greenhouses at rather low temperatures, high humidity, and high moisture content of the soil. Water is frequently sprayed. The experiments were performed with rooted cuttings.

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[1]) At the time the manuscript of this paper was ready to be sent to the printer a study of Göttgens appeared (GÖTTGENS, E. 1960. Untersuchungen über die Entwicklung von *Exobasidium Azaleae* Peck und seine Infektion der Wirtspflanzen unter besonderer Berücksichtigung der gallenbildenden Wirkstoffe. Phytopath. Z. **38**: 394–426). This author obtained pure cultures of the Azalea parasite in synthetic media and succeeded in infecting the host plant with basidiospores as well as with cell suspensions of pure cultures. Environmental conditions proved to be of influence on the length of the incubation period. Further Göttgens found that the culture filtrates contain activating substances, which promote the growth of tissue cultures of *Daucus carota* roots. As growth stimulating factors probably biotin, nicotinic acid or nicotinamide, inositol and pantothenic acid are present in the filtrate.

3. The first signs of attack by *Exobasidium japonicum* may appear even on the youngest just visible leaves of the unfolding buds. The diseased parts of the leaves are enlarged, and the blades become convex and crooked; these hypertrophic parts are called galls. In a young stage they are green, after ripening they become covered with spores, which give them a velvety appearance (Plate 1, A and B).

4. The dicaryotic, intercellular mycelium of the fungus forms basidia, which rupture the cuticle of the leaves. Each basidium bears four to five basidiospores (Fig. 1). The latter produce a great number of blastospores by budding. The tissue of the attacked parts of the leaves remains undifferentiated; both hypertrophy and hyperplasy of the mesophyll cells occurs (Fig. 2).

5. Colonies of *Exobasidium japonicum* were grown in pure cultures. As culture media malt and malt-saleb agar appeared to be most favourable. The fungus could also be grown in a malt solution in a shake culture. In both ways short hyphae and more or less regularly shaped, eventually fusiform cells, resembling blastospores were developed (Fig. 3).

6. The dormant buds were inoculated with basidiospores as well as with suspensions obtained from shake cultures. Simultaneously the shoots of the plants were decapitated in order to stimulate the development of the inoculated axillary buds. By this treatment the number of galls on the newly formed shoots appeared to increase considerably (Table 1). The source of the inoculum, whether basidiospores or cells from a shake culture, did not seem to influence the number of galls (Table 2).

7. The stage at which the fungus cells become dicaryotic was investigated. The basidiospores and the blastospores appeared to be uninucleate (Fig. 4 and 5). In vitro no cell fusion could be observed, neither between basidiospores nor between blastospores. Even between basidiospores and blastospores originating from one basidium, where in case of a sexual differentiation the presence of + and — spores could be expected, no fusion occurred (Fig. 6). The possibility, that cell fusion might take place on the surface of the leaf or after the fungus had penetrated into the leaf was also investigated. In that case at least one + and one — basidiospore, or a combination of their blastospores, would be necessary to accomplish infection. However, it appeared that blastospores originating either from a single cell out of a shake culture or from a single basidiospore were able to induce the development of galls (Table 3). Thus, it must be concluded that *Exobasidium japonicum* is homothallic.

8. By cross inoculations it appeared that *Exobasidium japonicum* did not attack *Vaccinium vitis idaea*, and that Azaleas were not attacked by *Exobasidium vaccinii* (Table 5). This difference in pathogenicity, added to a difference in colour of the colonies in vitro, are considered to be of specific importance, and for this reason *Exobasidium japonicum* Shir. is accepted as the valid name for the Azalea parasite. Strains of *Exobasidium japonicum* isolated from the Japanese Azaleas and from the cultivar 'Hexe', crossinoculated on the two host plants, proved to be identical in pathogenicity. (Table 4).

9. The leaves are probably susceptible to infection while still enclosed in the bud. By inoculating the leaves at different intervals after the buds began to unfold, i.e. after the tip of the shoot had been removed, it was shown that they remain susceptible until they have reached a length of 8–10 mm (Fig. 7, A-D; Plate 1, C-G and Table 6). In dormant axillary buds treated with a spore suspension, the fungus was still viable after 6 months, as after removal of the tip galls appeared on the unfolding leaves (Table 7).

10. The dormant buds should be protected by a fungicide against infection by wind-borne spores. Of the following fungicides different concentrations were assayed by adding them to a shake culture: copperoxychloride, lime sulphur, zineb and captan (Table 8). Captan strongly inhibited the growth of the mycelium at

a concentration of 0.2 g/l (Fig. 8). Buds on Azalea shoots were treated with this fungicide before, simultaneously with, and after inoculation with a spore suspension. The number of galls appeared to be most strongly reduced when the fungicide and the fungus inoculum were applied simultaneously (Table 9).

11. The similarity between the life cycle and the ecological behaviour of the genera *Exobasidium* and *Taphrina* is striking, though they are placed in quite different taxonomic groups, the *Basidiomycetes* and the *Ascomycetes* respectively. The relation of *Exobasidium* to the *Hemibasidiomycetes* is also discussed.

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SYMBIOTIC NITROGEN FIXATION IN NON-LEGUMINOUS PLANTS:

V. THE GROWTH REQUIREMENTS OF THE ENDOPHYTE OF ALNUS GLUTINOSA

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Introduction

Interest in symbiotic nitrogen fixation by non-leguminous plants has increased during recent years (we refer to the recent reviews of Allen and Allen 1959 and Bond 1958, 1959). The experiments done by Bond demonstrated the fixation of elementary nitrogen in root nodules of many non-legumes. It is, however, still impossible to cultivate the micro-organisms which are responsible for the formation of these nodules, even though they can be easily observed as endophytes in the nodular tissues. Many claims have been made that the endophyte of *Alnus glutinosa* had been isolated, but none of these claims has been able to stand the test of modern criticism. The only exception seems to be a very recent claim by Pommer (1959) that he had isolated an Actinomycete-like organism on plain glucose-asparagine-agar which was able to produce nodules on roots of *Alnus* plants under aseptic conditions. This claim by Pommer is so in contradiction to the experience of so many other investigators that his results are very difficult to reconcile with those of other experiments. We will try to do so after describing our own experiments.

These experiments were based on the idea that a reproducible isolation of the endophyte of *Alnus glutinosa* or those of other non-leguminous root nodules will only be possible when the growth requirements of these endophytes are sufficiently known. One of the previous communications of this series (1956) described a method which makes it possible to determine the increase of the number of infective particles in a nutrient solution during incubation after inoculation with a small amount of a suspension of "pure" nodules. It was then possible to determine the increase in the number of infective particles during a three-week period of incubation at 27° C. in certain peat suspensions, although no increase could be observed in other peat suspensions. It was shown that this difference was due to the presence of growth-inhibiting substances in the latter peat

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suspension and that the increase of the endophyte in the former peat suspension was caused by the presence of an alcohol-soluble growth-promoting substance (or substances). Addition of an alcoholic extract from these peats to a solution of glucose, peptone, yeast autolysate, and salts gave a small development of the endophyte in these solutions as measured by the increase of the infective capacity during incubation. The same result was obtained after addition of an alcoholic extract of N-deficient alder roots. Without these extracts or with water extracts of peats or roots no development was observed.

However, in all these experiments the results appeared to be badly reproducible. This was partly caused by the presence of growth-inhibiting substances in some alcoholic root extracts, but further experiments showed that other causes were responsible as well. Theoretically, three types of factors may be responsible for this unreproducibility:

1. differences in the composition of the basal nutrient solution,
2. differences in the composition of the alcoholic root extracts,
3. differences in the endophyte content and the chemical composition of the nodules used for the inoculation.

It took a long time to unravel the causes of the unreproducibility since so many causes were simultaneously operative that few experiments gave reliable information. However, this information was not only important for elucidation of the causes of the unreproducibility but also for a better understanding of the growth requirements of the endophyte.

In the following article we will give a survey of some crucial experiments which demonstrate the role of growth-promoting and growth-inhibiting substances and the first efforts to identify the chemical nature of the growth-promoting substance in the alcoholic root extracts.

Methods

The methods used for the determination of the growth of the endophyte in vitro were essentially the same as those previously used (Quispel 1954, 1956). We will therefore restrict ourselves to the decription of the main principles and certain improvements in the method.

a. Preparation of the inoculation

Inoculation was effected by a suspension of nodules which had been purified by the method of selective incubation. During the selection, most of the nodules had to be discarded because they were infected by contaminating organisms. This number could be considerably reduced by a second disinfection of the nodules after the removal of the outer cell layers. The second disinfection did not affect the vitality of the endophyte inside the nodules. The present procedure is as follows: nodules are sampled in the field, washed with water, soap and alcohol and, after rinsing with water, disinfected

with a bromium solution (0.1 % v/v) for 5 minutes. After four successive washings with sterile water the outer cell layers are removed with a sterile scalpel. The peeled nodules are collected in a petri dish containing sterile water and finally again disinfected with bromium for a few minutes. The nodules then are washed with four successive portions of sterile water and each nodule is put in a tube with glucose-peptone-yeast autolysate-agar. After six weeks incubation at 27° C. the infected nodules are discarded and the nodules which on careful examination appear to be sterile are crushed in sterile water. In all experiments, 10 nodules were crushed in 5 ml sterile water with a sterilized glass rod in a thick-walled culture tube. After crushing, the suspension was allowed to stand for a few minutes so that the heavier particles could sink to the bottom of the tube. The supernatant was then decanted and used for the inoculation of the culture media. One drop of this suspension (0,05 ml) was inoculated with a sterile pipette into 10 ml nutrient solution.

b. Nutrient solutions

The composition of the basal nutrient solution is: water 1000 ml, K_2HPO_4 300 mgm, NaH_2PO_4 200 mgm, $MgSO_4 . 7H_2O$ 200 mgm, KCl 200 mgm, $CaCO_3$ 2 gm, glucose 20 gm, peptone Fleisch Merck 5 gm. In the first experiments, NH_4NO_3 500 mgm and 10 ml yeast autolysate were added but these additions were later omitted. All chemicals were ANALAR quality in double glass-distilled water. Culture tubes of 16 mm diameter were provided with 10 ml of these nutrient solutions and sterilized by autoclaving for $\frac{1}{2}$ hour at 120° C. Where filtration was used for sterilization a Jena G5f or a Seitz filter was employed. In this case the salts and the alcoholic extracts (see below) were dissolved in 5 ml water and sterilized by autoclaving while the glucose and peptone were added in 5 ml portions after filtration.

c. Preparation of the alcoholic root extracts

If not otherwise specified, the alcoholic root extracts were prepared from roots of 14 week old *Alnus* plants which had been cultivated on the normal HOAGLAND nutrient solutions during the first 8 weeks and on a N-free nutrient solution during the last 5–6 weeks (nitrates replaced by equivalent amounts of the chlorides). The roots were separated from the shoots, washed, dried in vacuo at 55° C., powdered in a mill, and stored as a dry powder.

Before the alcoholic extraction the powder was repeatedly extracted by boiling with water and centrifugation of the extract at 12000 g. This procedure was repeated till the supernatant was only faintly coloured. The extracted sediment was again dried in vacuo and then extracted with 95 % alcohol. This alcoholic extract was diluted in such a way that 1 ml corresponded to the equivalent of the dry matter of one root system (± 500 mgm).

A 1 ml. aliquot of this extract was added to 10 ml nutrient solution. Because the alcohol must be completely removed and the dissolved

substances finely dispersed in the nutrient solution, the extracts were added in the following way: 1 ml extract was pipetted into a tube with a few drops of water. The tube was put into a boiling water-bath till all the alcohol was evaporated and the dissolved lipids formed a cloudy suspension in the remaining drops of water. The nutrient solution was then added to the tubes. Since the insoluble residue cannot be filtrated, this residue of the extract was always sterilized by autoclaving together with the salt solution.

d. Purification and fractionation of the alcoholic extracts

1. *Preparation of the petroleum ether fraction*

The alcoholic extract was evaporated in vacuo and dried. The dry residue was repeatedly extracted with petroleum ether (boiling point 40–60° C.), and the petroleum ether solutions filtered and combined. The filters were washed with petroleum ether and this petroleum ether added to the other petroleum ether extracts. The petroleum ether solution was evaporated at 55° C. in vacuo and the residue taken up in 96 % alcohol and brought back to the original volume.

2. *Separation of the acid from the non-acid lipid fractions*

A 10 ml aliquot of extract was evaporated in vacuo and dissolved in petroleum ether as described above. The petroleum ether solution was extracted with 10 % KOH in water in a separation funnel. The KOH layers were again extracted with petroleum ether. The separation of the layers was very difficult because a foamy precipitate formed between the two layers. This precipitate was collected in the alkaline water fraction which developed a cloudy appearance. The alkaline layer could only be cleared by extraction with diethyl ether. The diethyl ether solution was added to the petroleum ether layers. The ether-petroleum ether layers were again extracted with distilled water and the water added to the alkaline fraction. The ether-petroleum ether layers were evaporated and dissolved in 96 % alcohol and brought back to the original volume of 10 ml. This solution contained the non-acid lipids.

The alkaline water layers were acidified with HCl and extracted with petroleum ether. The remaining solution was evaporated in vacuo to dryness and the dry residue again extracted with petroleum ether. The petroleum ether solutions were added together, evaporated, and the residue dissolved in 10 ml of 96 % alcohol. This solution contained the fatty acids.

3. *Hydrolysis of the alcoholic extracts*

A 20 ml aliquot of extract was evaporated in vacuo and dissolved in 20 ml 10 % KOH in methanol. The solution was boiled under reflux for 4 hours, 5 ml water was then added, and the boiling continued for another 2 hours. The next morning the precipitation was filtered, dissolved in 6 % KOH in methanol, and boiled for 3 hours. The now clear solution was added to the filtrate, acidified, extracted

with petroleum ether as described above and finally the petroleum ether extract was transferred to 96 % alcohol.

4. *Chromatographic fractionation of the alcoholic extracts*

Chromatographic analysis of the alcoholic extracts was performed with a modification of the methods of FILLERUP and MEAD (1953) and HIRSCH and ARENS (1958). The columns were 2 cm in diameter and 7 cm in height, filled with silicagel (Light 100–200 mesh). The silicagel was purified according to Hirsch and Arens, and activated at 115° C. over night. The dry silicagel was brought into the adsorption tube, washed with petroleum ether and thoroughly mixed with the petroleum ether so that the particles sedimented in the tube. When nearly all the petroleum ether had passed the column so that the surface was nearly dry, 10 ml petroleum ether extract was brought on the column and washed away with another portion of 10 ml petroleum ether. The column was then eluted with successive portions of 100 ml of 1 % ether in petroleum ether (4 portions), 4 % ether in petroleum ether (4 portions), 10 % ether in petroleum ether (4 portions) 50 % ether in petroleum ether (3 portions), pure ether (3 portions) and methanol (2 portions). All eluates were transferred to alcohol in the usual way.

e. Incubation

Unless not otherwise specified, all inoculated tubes with nutrient solution were incubated at 27° C. for three weeks. From time to time the tubes were shaken by hand. The eventual growth of the endophyte in these tubes was determined by the determination of the infective capacity of the tubes before and after incubation.

f. Determination of the infective capacity of the tubes

The infective capacity of the nutrient solutions before and after incubation was determined by bringing known amounts of the solutions into jars with *Alnus* plants which had been transferred to a N-free nutrient solution. The details of this procedure were exactly the same as those described in the first, second and third communication of this series (1954, 1956), The total amount of endophyte in a solution was calculated in relation to the amount used in the standard experiment described in these former communications and expressed as the Endophyte Concentration (EC).

In many experiments it was sufficient to compare the growth in different nutrient solutions which were simultaneously inoculated with the same amount of one pure nodule suspension. If these nutrient solutions are incubated under exactly identical circumstances, it is sufficient to determine the amount of nodules which are formed by *Alnus* plants after inoculation with the same amount of these different inoculated and incubated nutrient solutions. These amounts must be chosen in such a way, that, if no growth of the endophyte occurred during incubation, practically no nodules are formed on the roots of the plants. On the other hand, the amounts used for the inoculation

of these plants must be so large that a considerable number of nodules are formed if some multiplication of the endophyte occured during the incubation period. According to our experience, the following procedure always gave reliable results. The incubated nutrient solution, inoculated with the amounts described above, was diluted to 100 ml, and from this dilution 5 ml was added to jars with 350 ml N-free nutrient solution and three 7 week-old *Alnus* plants per jar. If no growth occurred during incubation the number of nodules counted on these plants never exceeded 1 or 2. If growth of the endophyte occurred during the incubation period, the number of nodules on the plants varied between 5 and 50. In most of the Tables this number of nodules is used as a direct indication for the growth in vitro of the endophyte during incubation.

g. Control of sterility

It appeared that the faint growth in vitro of the endophyte which can be determined with this sensitive method is very susceptible to the presence of contaminating organisms. All tubes with inoculated and incubated nutrient solutions were tested for the presence of contaminating organisms by the inoculation of tubes with peptone-glucose-yeast autolysate-agar, where the endophyte itself does not show any development. All tubes which appeared to contain other organisms were discarded.

Results

a. The composition of the basal nutrient solution

1. *The presence of yeast autolysate*

One of the possible causes of unreproducibility might have been the yeast autolysate with its unknown, complex, and uncertain composition. It appeared that the addition of yeast autolysate could be omitted for at least the first inoculation. Moreover, it was shown by some experiments that sometimes yeast autolysate may exert a growth-inhibiting effect. This is apparent from the results of the experiment described in Table 1.

Table 1

Influence of peptone and yeast autolysate on the growth in vitro of the endophyte.

The nutrient solutions contained the usual salts, glucose, and an alcoholic root extract. The solutions differed as to the presence of peptone and yeast autolysate. The incubated nutrient solutions were used to inoculate 4 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

concentration of peptone	concentration of yeast autolysate	
	0	1 ml %
0	0.2	0
0.5 %	17.8	0.1

Although in other experiments the inhibiting influence of yeast autolysate was not as pronounced as in this one, it was considered advisable to omit the yeast autolysate in the nutrient solution of future experiments.

2. *The necessity of peptone*

The results shown in Table 1, suggest that another conclusion may be drawn because peptone appears to be an indispensable ingredient of the nutrient solution. Since there are important differences between different samples of peptone, the addition of peptone was standardized by always using the same type and quality (peptone Fleisch Merck). This of course does not mean that other samples would give less consistent results.

In the contradictory experiments done by Pommer, asparagin was used instead of peptone. It was therefore interesting to compare the effect of asparagin with the effect of peptone. This was done in the experiment summarized in Table 2.

TABLE 2

Influence of peptone and asparagin on the growth in vitro of the endophyte.

The nutrient solutions contained the usual salts, omitting NH_4NO_3. Glucose and peptone or asparagin were sterilized by filtration. Half of the solutions contained an alcoholic root extract purified with petroleum ether.

The experiment was performed in duplicate. The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Concentration of peptone	Concentration of asparagin	alc. root extr.	Number of nodules in series A	Number of nodules in series B
0.5 %	0	0	0.2	0
0.5 %	0	0.1 ml %	10.2	6.7
0	0.1 %	0	0	0.2
0	0.1 %	0.1 ml %	0.3	0.8

It is evident that asparagin cannot replace peptone. As in all the other experiments, an indication for growth in vitro was only obtained when the alcoholic root extract was added to the solutions.

3. *The source of carbon*

In the basal nutrient solution glucose was used as a source of carbon. Many of the nutrient media used for the cultivation of *Actinomycetes* contain glycerol. According to cytological observations, starch is the main reserve substance in the *Alnus* roots and most probably the natural carbon source for the endophyte. In the experiment shown in Table 3 these three carbon sources are compared.

It is evident that in this experiment only starch is a suitable source of carbon. This is very remarkable, because in other experiments glucose enabled the development of the endophyte.

TABLE 3

Influence of the source of carbon on the growth in vitro of the endophyte.

The nutrient solutions contained the usual salts with peptone. The complete nutrient solution including the sources of carbon was sterilized by autoclaving. The experiment was performed in quadruple. The four series contained different alcoholic root extracts, all purified with petroleum ether.
The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Source of carbon	Number of nodules in Ser. A	Ser. B	Ser. C	Ser. D
2 % glucose	0	0	0	0
2 % glycerol	0	0	0	0
2 % starch (soluble) . .	14.2	16.8	14.5	4.7

4. *The sterilization of the nutrient solution*

The discrepancies between different experiments where glucose was used as a source of carbon might be explained by the formation of inhibiting substances during the autoclaving of glucose. It is possible that in some experiments these substances were present in such quantities that their inhibiting effect was observed, while in other experiments their presence was not injurious. In the experiment shown in Table 4, a comparison was made between solutions sterilized by autoclaving and solutions in which the glucose and peptone were sterilized by filtration.

TABLE 4

Influence of the method of sterilization of the glucose and peptone in the nutrient solution on the growth in vitro of the endophyte.

The nutrient solution contained the usual salts with glucose and peptone. The experiment was performed in duplicate with two different alcoholic root extracts purified with petroleum ether.
The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules per plant.

Method of sterilization	Number of nodules in Ser. A	Ser. B
Autoclaving	0	0
Filtration of glucose and peptone, autoclaving of salts and alc. extract.	24.8	21.8

It appears that sterilization by filtration enabled the growth of the endophyte, while growth was impossible in the solutions where the glucose and peptone had been sterilized by autoclaving. We must conclude that sterilization by autoclaving induces the formation of substances which are inhibitive to the growth of the endophyte. In subsequent experiments glucose and peptone were always sterilized by filtration.

5. *The influence of other factors*

The pH of the nutrient solution after sterilization was 6.8. By addition of NaOH or HCl, slight variations in pH were obtained. A

somewhat more acid pH (6.0) gave the same results, but a slightly alkaline pH (7.6) gave somewhat less growth.

In one experiment, omission of NH_4NO_3 had a favourable effect. Though this was never observed in later experiments no NH_4NO_3 requirement could be demonstrated and in future experiments this salt was omitted.

6. *Conclusion*

The best results were obtained with the basal nutrient solution if yeast autolysate was omitted, peptone was present, and glucose and peptone were sterilized by filtration.

b. The effect of the alcoholic root extracts

In all the nutrient solutions which were used in the experiments described above, growth of the endophyte was only observed if an alcoholic root extract was added to the nutrient solutions. The improvement of the basal nutrient solution was only effective if this extract was present; without this extract no traces of growth were ever observed. The study of this extract is certainly one of the most important aspects of the study of the growth-requirements of the endophyte.

1. *The effect of alcohol*

It is possible that the effect of the alcoholic extracts was caused by the alcohol itself. This supposition was very improbable because the alcohol was damped off in a boiling water bath, but some residual alcohol might have influenced the results. This was checked in some experiments in which a small amount of alcohol was added after sterilization to samples with and without the alcoholic extract. One of these experiments is summarized in Table 5.

TABLE 5

Influence of alcohol on the growth in vitro of the endophyte.

The nutrient solution contained the usual salts plus glucose and peptone sterilized by filtration. Half of the solutions contained an alcoholic root extract purified with petroleum ether. The alcohol was added after sterilization. The experiment was performed in duplicate. The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Concentration of alcohol	with alc. root extract		without alc. root extract	
	Ser. A	Ser. B	Ser. A	Ser. B
0	15.8	25.5	0.8	1.0
0.001 ml %	7.7	12.0	1.5	1.2
0.01 ml %	3.7	3.5	0.5	1.3

There is no indication that alcohol promotes the growth of the endophyte. This is the more evident because in nutrient media containing inhibitive concentrations of alcohol, growth was nevertheless

stimulated by the addition of the alcoholic root extract. Clearly the growth-promoting influence of the alcoholic root extract is not caused by retention of small amounts of alcohol in the nutrient solution. Small amounts of alcohol definitely inhibit growth and might have been partly responsible for the unreproducibility of some experiments. It is advisable to remove the alcohol as thoroughly and carefully as possible.

2. *The removal of inhibiting substances*

In a previous communication of this series, experiments were described which demonstrated the possible presence of toxic substances in the alcoholic root extracts. This was certainly one of the main reasons for the unreproducibility of the first experiments. Therefore it was very important to separate the growth-inhibiting substances from the growth-promoting factors. It was possible to do this very simply, by fractionation with petroleum ether. A comparison between the effects of normal and purified extracts is given in Table 6.

TABLE 6

Effect of the purification of the alcoholic root extracts with petroleum ether on their promotion of growth of the endophyte in vitro.

The nutrient solutions contained the usual salts plus glucose, peptone, and yeast autolysate, all sterilized by autoclaving.

The experiment was performed in duplicate with two different alcoholic extracts. Both extracts were added either directly or after purification by transfer to petroleum ether.

The incubated nutrient solutions were used to inoculate 4 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Fraction of extract	Number of nodules in Ser. A	Ser. B
complete extract	17.3	0
petroleum ether-soluble fraction	29.1	18.0
water soluble fraction	0.6	0

Table 6 shows that the growth-promoting substances or substance are still present after the fractionation in the petroleum ether solution. Moreover, it appears that an inactive extract may become active after this purification. Obviously the growth-inhibiting substance or substances are not soluble in petroleum ether. This observation has considerably increased the reproducibility of later experiments. Moreover, an important conclusion may be drawn: the growth-promoting substance is lipophilic. This was further confirmed by experiments where the solution in petroleum ether was extracted with water in a separation funnel without loss of activity.

3. *Further chemical fractionation of the extract*

Since it was shown that the growth-promoting substances belongs to the lipids, the next step was to find out whether the growth-promoting substances belonged to the hydrolysable lipids, to the fatty

acids, or to the neutral lipids. This was done with the usual methods of hydrolysis and extraction with alkali. The results of an experiment of this type are shown in Table 7.

TABLE 7

Effect of alkali-extraction and alkaline hydrolysis of the alcoholic root extracts on their promotion of growth of the endophyte in vitro.

The nutrient solutions contained the usual salts excepting NH_4NO_3, plus glucose and peptone sterilized by filtration, and different fractions of the alcoholic root extract.
The incubated nutrient solutions were used to inoculate 2 jars containing 2 plants each. The figures refer to the average number of nodules formed per plant.

	Normal extract	Saponified extract
unfractionated	1.3 (?)	8.7
petroleum ether layer after KOH extraction	44.2	13.9
KOH extract	0	—

For some unknown reason, in spite of purification with petroleum ether the growth in the tube with the untreated extract was very small. However, it is evident that the growth-promoting substance is resistant to hydrolysis and is not extracted with alkali either before or after hydrolysis. The growth-promoting substance therefore belongs to the non-acid, non saponifiable lipids.

4. *Chromatographic separation*

Though the efforts to isolate the growth-promoting substance are still in a very early stage, it is worthwhile to give a preliminary account of the first results.

Some older experiments have shown that the growth-promoting substance is not adsorbed to the kation exchanger Imac C 12 and the anion exchanger Imac A 17 (Activit, Amsterdam) from a 70 % alcohol solution. This is in accordance with the conclusion from Table 7, that the substance belongs to the neutral lipids.

Some preliminary experiments were performed with Aluminium-oxyde Brockmann (Merck). The substance is adsorbed to this adsorbens from petroleum ether and eluted with 50 % ether in petroleum ether.

The most extensive experiments were performed with silicagel as adsorbing substance. The result of one of these experiments is given in Table 8.

The substance is adsorbed to silicagel from the petroleum ether solution. In 1 % and 4 % ether in petroleum ether, no elution is observed. Small amounts are eluted with 10 % ether, 50 % ether, and 100 % ether, but the bulk of the substance is eluted by methanol. Most probable the results are influenced by secondary adsorption and elution, since the growth-promoting substance is certainly present together with far greater amounts of other lipids. Better results may be obtainable by chromatography after a previous chemical purification.

Table 8

Growth-promoting effect of eluates from a silicagel column, on which the petroleum ether solution of an alcoholic root extract had been adsorbed, on the growth of the endophyte in vitro.

The nutrient solutions contained the usual salts excepting NH_4NO_3, glucose and peptone sterilized by filtration, and equivalent amounts of the eluates. The incubated nutrient solutions were used to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

Eluate	Number of nodules in successive fraction				Moment of elution of some pure lipids
	1	2	3	4	
1 % ether/petr. ether .	0	0	0.3	0.3	carotene
4 % ether/petr. ether .	0.5	0	0.7	0	
10 % ether/petr. ether .	6.0	4.7	8.7	6.6	palmitic acid
50 % ether/petr. ether .	12.8	6.7	5.3	—	cholesterol
ether	10.8	1.5	2.8	—	
methanol	36.5	2.3	—	—	

5. *The physiological condition of the roots before the extraction*

All alcoholic extracts were prepared from roots which had been cultivated on a N. free nutrient solution for at least 5 weeks. This procedure was inspired by the suggestion that the growth-promoting substances might be most abundant in N-deficient roots because in vivo the formation of root nodules, and thus the growth of the endophyte, is promoted by N-deficiency of the roots. Some experiments were carried out to compare the growth-promoting activity of extracts prepared from roots with different degrees of N-deficiency with roots cultivated exclusively on the normal nutrient solution. It appeared that the growth-promoting substances can be extracted from all roots, while the differences between the growth-promoting effect of extracts from normal and N-deficient roots were too small to allow of any conclusions.

6. *Addition of known substances*

In the course of the experiments many efforts were made to replace the alcoholic extract by known alcohol-soluble substances. No effect was obtained with cholesterol, ergosterol, vitamin D1, vitamin D2, tocopherol, menadion, lecithin, indole acetic acid, oleic acid or olive oil.

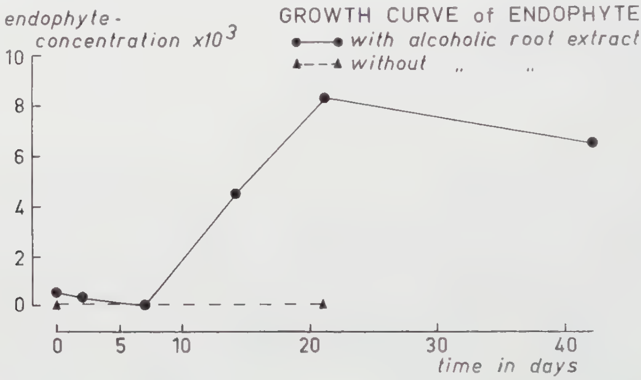
7. *Conclusion*

Reliable and reproducible growth-promoting effects are obtained by alcoholic extracts of *Alnus* roots if these alcoholic extracts are fractionated by transfer to petroleum ether. The active substance belongs to the non-acid, non-saponifiable lipids, is not adsorbed to ion-exchangers, but is adsorbed to aluminiumoxyde and silicagel. The activity could not be replaced by some known lipids.

c. The growth curve of the endophyte in vitro

In all previous experiments growth was determined after an arbitrarily chosen incubation period of three weeks. As soon as the

growth requirements were sufficiently analyzed to make reproducible experiments possible, the growth curve of the endophyte in vitro was determined in a nutrient solution without NH_4NO_3, without yeast autolysate, with the glucose and peptone sterilized by filtration, and with the petroleum ether fraction of an alcoholic root extract. A number of tubes containing this nutrient solution were simultaneously inoculated, and at intervals one of these tubes was used for the determination of the Endophyte Concentration. The results are given in Fig. 1. The same Figure gives a growth curve drawn from an experiment in which the alcoholic root extract was omitted.



In the solution without root extract, no growth was observed, as usual. In the solution containing alcoholic root extract, growth started during the second week of incubation and reached an optimum during the third week. The small decrease during the first week and after the third week may be regarded not significant. The determination of growth after three weeks' incubation appears to have been a very lucky choice.

d. The transfer from incubated nutrient solutions

In the Introduction, another possible cause of the unreproducibility of the growth experiments was mentioned: the nodules with which the tubes were inoculated. These nodules were always sampled in the field and, in consequence, must have been different from one experiment to another. There may have been differences in the content of endophyte and in the vitality of the suspension used for the inoculation. Since a certain amount of nodule material is always inoculated together with the cells of the endophyte, substances may be present that either decrease or increase the possibilities for growth in the nutrient solutions. Finally, the preparation of pure nodules by the method of selective incubation has the disadvantage that some slow-growing contaminating organisms may still be present. All these factors remain causes of unreproducibility which can only be overcome by duplicate or triplicate experiments, or by inoculations from nutrient solutions in which growth of the endophyte has been demon-

strated. Unfortunately, transfers from growing cultures into new nutrient solutions failed in nearly all cases. Only in a very few experiments could growth after transfer be demonstrated.

This failure of the transfers can be explained in several ways. It is possible that the nodule material which is inoculated together with the endophyte contains some indispensable growth substances. In that case transfers will be possible if the nutrient solutions contain endophyte-free nodule material. In a preliminary experiment which must be repeated for further evidence, an indication was obtained that this is indeed the case. In this experiment, which is summarized in Table 9, a number of tubes were each inoculated with one drop from an inoculated and incubated nutrient solution. Some of these tubes were provided with a water extract of peeled nodules, centrifuged at 16000 g., and sterilized by filtration through a Jena G5f filter. Other tubes were provided with some well-known vitamins.

TABLE 9

Growth of the endophyte after transfer from one culture to another nutrient solution.

Nutrient solutions with the usual salts excepting NH_4NO_3, with glucose and peptone sterilized by filtration, and with the petroleum ether fraction of an alcoholic root extract, were inoculated with a pure nodule suspension. After three weeks' incubation, one drop of this solution was inoculated in fresh nutrient solution with the same composition but either with or without the alcoholic root extract. Some tubes of the latter solution were provided with 1 ml of an extract from peeled root nodules (10 gram fresh weight in 100 ml water) sterilized by filtration.

Other tubes were provided with 0.1 ml per 10 ml of the following vitamin solution: 500 ml water, 50 mgm inositol, 10 mgm Ca panthothenate, 10 mgm thiamin, 10 mgm niacin, 10 mgm pyridoxin, 2 mgm p. aminobenzoic acid, 0.025 mgm biotin.

The last series of nutrient solutions was used after incubation to inoculate 2 jars containing 3 plants each. The figures refer to the average number of nodules formed per plant.

	with alc. root extract	without alc. root extract	not inoculated
normal medium	0	0	0
with nodule extract	8.8	0	0
with vitamin solution . . .	0	0	0

It is evident from the results of this experiment that growth in a nutrient solution after transfer from another culture was only possible when the second solution contained the alcoholic root extract together with the water extract of the nodules. Since the same solution inoculated with a drop of sterile nutrient solution did not give any development, it is improbable that this result was caused by the presence of vital cells off the endophyte in the filtered nodule extract. Moreover, in former experiments no indications were obtained that the endophyte can pass bacterial filters. The conclusion seems warranted that the nodules contain an indispensable, as yet unidentified, growth substance which is present in the nodule material of the first inocula-

tion but has to be added to the nutrient solution if transfers to fresh nutrient solutions are made. This substance is not identical with one of the added vitamins.

e. The direct microscopic observation of the endophyte

The time-consuming method used in the experiments described above is only necessary when the growth of the endophyte in vitro is so small that a more direct observation is impossible. In recent experiments the growth as measured by the increase of the infective capacity, was so remarkable that it was asked whether the cells of the growing endophyte could not be seen under the microscope. In a very infective culture, clusters of very thin hyphae were observed. These *Actinomycete*-like organisms have hyphae with a diameter of less than 1 μ, with a granulated appearance. In one case it was observed that these hyphae grew out of a piece of nodule tissue. Though we must be very cautious because contaminations are always possible, it is very probable that these clusters of hyphae are indeed the cells of the organisms which are responsible for the infectivity of the solution.

Discussion

When considering the results of our experiments we must thoroughly realize the special character of the method used as described in a previous communication (1956). We should realize that this method is an indirect determination of growth in which the growth of the endophyte in vitro is measured by means of the increase of the infectivity of an inoculated nutrient solution during incubation. This infectivity is measured by counting the number of nodules formed in water cultures of *Alnus glutinosa* when these cultures are inoculated with known amounts of the inoculated and incubated nutrient media. The number of nodules depends on the number of infective particles. In consequence, the method determines the increase in the number of infective particles during incubation. We must realize that no growth is observed if the endophyte loses its virulence during growth: only the infective cells are determined. Moreover, the method is essentially a method for the determination of cell divisions. A mere fragmentation of hyphae will be measured as growth, while an extension of cells without any cell divisions can not be observed.

With these restrictions in view, it appears that the experiments have again shown the absolute necessity of an alcohol-soluble substance for the growth of the endophyte. This necessity was evident in all nutrient solutions used, in no case was any trace of growth observed if no extract was present. The activity of this extract is caused by a non-acid, non-saponifiable lipid. The bad reproducibility of the first experiments appeared to have been due to the possible presence of inhibiting substances in the unpurified root extracts, in the yeast autolysate, and in the autoclaved glucose solutions. Better results were obtained if the alcoholic extracts were purified by transfer

to petroleum ether, when the yeast autolysate was omitted, and when the glucose and peptone were sterilized by filtration. Some indications were obtained for the presence of another indispensable substance in the root nodules used for the inoculation. Peptone is indispensable and can not be replaced by asparagin.

The difficulties with which so many investigators have struggled in their vain efforts to isolate the endophyte appear to have been caused by the influence of the inhibiting substances together with the need for the growth-promoting lipoid substance, and by the very slow growth rate and minimal development even when the circumstances are favourable for growth.

These results are completely contradictory to those which POMMER (1959) published after the experiments described above were finished. According to Pommer, the endophyte grows on plain glucose-asparagin-agar without further additions, though growth is stimulated by the addition of a water extract of peeled *Alnus* nodules. When pieces of nodules, disinfected with phenole, were inoculated on glucose-asparagin-agar, growth of an *Actinomycete*-like organism was observed after several days. This claim has been made by many authors, but Pommer has demonstrated the nodulating capacity of this organism in a reproducible way under aseptic conditions. The description of his experiments does not allow of any criticism, they deserve serious consideration and urgently need repetition.

On some preliminary experiments we have tried to repeat his experiments and have in some cases indeed observed organisms of the type he described, though only on media with nodule extract and after a much longer incubation period. The very thin hyphae resemble the very thin hyphae which were found in our infective nutrient solution.

There would be no difficulty in accepting Pommer's results if the growth requirements of his organism were not much simpler than those indicated by our experiments. If, indeed, his organism turns out to be the endophyte, how is it then possible that his organisms grows on plain glucose-asparagin media while our experiments have without exception demonstrated the necessity for both a lipophilic substance and peptone for even a very faint development? It seems reasonable to explain this discrepancy in terms of the difference in the methods used. Pommer directly observed growing hyphae while we measured the increase in infective capacity. He determined growth of cells while we could only observe the increase in number of infective particles. As has been stressed above, we might have overlooked growth of the endophyte if no cell-divisions had occurred. It is improbable, however, that this is the reason for the discrepancy. If clusters of hyphae grow in length, the chance for subsequent fragmentation during shaking of the suspension of these clusters will certainly increase (before inoculation of the *Alnus* cultures the tubes were heavily shaken). A more reasonable explanation may be found in the infective capacity of the growing cells. According to a personal communication from Pommer, he has observed still more rapidly growing cultures

of an *Actinomycete* which might be identical with the non-infective type of organism that has been isolated many times before. It then might be possible that the organism occurs in different forms (mutants? modifications?): a rapid-growing, non-infective form with simple growth requirements and a slow-growing, virulent form with complex growth requirements (or requirements to remain in the virulent form). In our experiments only the growth requirements of the latter form could be determined. Pommer may have isolated a kind of intermediate form with simple growth requirements but which has retained some of its virulence. Another explanation might be that his cultures consist of a symbiosis between the rapid-growing *Actinomycete* with the virulent endophyte. These are of course mere speculations, but these speculations may be fruitful working-hypotheses for attempts to reconcile Pommers' results and ours, and to stimulate further research in this field.

SUMMARY

The growth in vitro of the endophyte of *Alnus glutinosa* is studied by determining the increase of the infective capacity of inoculated nutrient solutions during incubation. Growth is only possible if an alcoholic extract from *Alnus* roots is added to the nutrient solutions. This extract may also contain inhibiting substances but these substances are removed by transfer to petroleum ether. The growth-promoting substance belongs to the non-acid, non-saponifiable lipids. The substance is not adsorbed to ion-exchange resins but is adsorbed from the petroleum ether solution by aluminiumoxyde or silicagel. The growth of the endophyte is inhibited by substances formed during autoclaving of glucose and by yeast autolysate. Peptone is indispensable and can not be replaced by asparagin. Some indications were obtained that growth is stimulated by a water-soluble factor present in *Alnus* nodules. Even under favourable conditions the growth rate is very low, maximum growth being obtained after 2–3 weeks. In an infective culture, clusters of very thin hyphae were microscopically visible.

The results are discussed with regard to the conflicting results obtained by Pommer and a working-hypothesis is suggested to reconcile the different observations.

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PROSOPIS INSULARUM (GUILL.) BRET., A NEW COMBINATION IN PROSOPIS L. (MIM.)

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(*received July 19th, 1960*)

A study of the species of *Leucaena* Bth. and their hybrids as cultivated in Netherlands New Guinea, revealed that the systematical status of *Leucaena insularum* (Guill.) Dän. needed to be re-examined. *L. insularum* was first described by GUILLEMIN in 1837 and based on a specimen from Tahiti. He named it *Acacia insularum* Guill. In 1932, DÄNIKER placed *A. insularum* in *Leucaena* and published the combination *Leucaena insularum* (Guill.) Dän. BENTHAM had already placed the same species in *Leucaena* in 1846 and then named it *Leucaena forsteri* Bth.

It appeared that some specimens kept in the Leiden Herbarium, collected in New Guinea, the Solomon Islands, and the Moluccas, which had been identified as *Leucaena* sp., belonged to a taxon that was either identical with, or closely allied to, *Leucaena insularum* (Guill.) Dän. Dr. R. D. HOOGLAND (C.S.I.R.O. Herbarium, Canberra) supplied information which led to the identification of the specimens as *Piptadenia novo-guineensis* Warburg. WARBURG's detailed description made it possible to identify the specimens with certainty.

A wider range of specimens was examined and it finally appeared that both *Leucaena insularum* (Guill.) Dän. and *Piptadenia novo-guineensis* Warb. belonged to the same genus, but neither to *Leucaena* nor to *Piptadenia*.

The anthers in *L. insularum* and in *Piptadenia novo-guineensis* are glanduliferous and the seeds contain endosperm; both these characters are not admissible in either *Leucaena* or *Piptadenia* (according to some authors a trace of endosperm may be seen in the seeds of some species considered to belong to *Piptadenia*). The pods of both taxa have a distinct mesocarp and well-marked septs. They seemed to be indehiscent. WARBURG, however, described (l.c. 336) the pods as dehiscent, but I never found a mature dehiscent pod on any specimen I examined. Young and immature pods, on the other hand, after being dried, sometimes appear to be dehiscent. The pods, apparently, shed the seeds after the margins become detached or split as far as to open the seed-containing loculi (see fig. 2:6). They do not open further and it can be said that the pods are indehiscent, although the gaping margins suggest dehiscence. A similar opening of the pods occurs e.g. in *Cassia alata*. On the strength of these three characters (glanduliferous anthers, indehiscent pods, and albuminous seeds), it seems best to refer both *L. insularum* (Guill.) Dän. and *Piptadenia novo-guineensis* Warb. to the genus *Prosopis* L.

One of the most important differences between these two taxa can be observed in the seeds. The seeds of western specimens (distributional area the Moluccas to the Solomon Islands) are glossy and very often narrow. The seeds are dull and mostly broader in eastern specimens (distributional area the New Hebrides and New Caledonia to Tahiti). Other characters of Western specimens are more seeds to the pod and more jugae to the leaf, but though probably statistically a difference could be shown to exist, these characters are not constant.

These results (small morphological differences combined with geographical segregation) indicate that it is not advisable to maintain WARBURG's taxon as a distinct species. It is a subspecies of GUILLEMIN's species, originally described as *Acacia insularum.*

Mrs. Dr. J. A. FRAHM–LELIVELD of the Laboratory of Tropical Agriculture who kindly examined the cytology of young seedlings, found for an eastern specimen chromosome number 2n = 52 (Tonga Islands, Yuncker 15108), and 2n = 54 for a western specimen (New Guinea, Lam/Versteegh BW 750).

I am much indebted to the Directors or Keepers of the following Herbaria for the loan of specimens, or for making specimens available for examination, or supplying information: Kew (K); Leiden (L); London (BM); Paris (P); Utrecht (U); Wageningen (WAG); Zürich (Z).

Prosopis insularum (Guill.) Bret. comb. nov.

Acacia insularum Guillemin, Ann. Sc. Nat. Ser. II, 8:360. 1837.
Leucaena insularum (Guillemin) Däniker, Vierteljahrschr. Nat. Ges. Zürich 77 (Beibl. 19):176. 1932; Yuncker, Plants of Tonga, B.P. Bishop Mus. Bull. 220:130. 1959.
Leucaena forsteri Bentham, London Journ. Bot. 5:94.1846.
Mimosa glandulosa Solander ex Forster, Prodr.: 92. 1786; nomen nudum.

——— subsp. **insularum** Fig. 1.

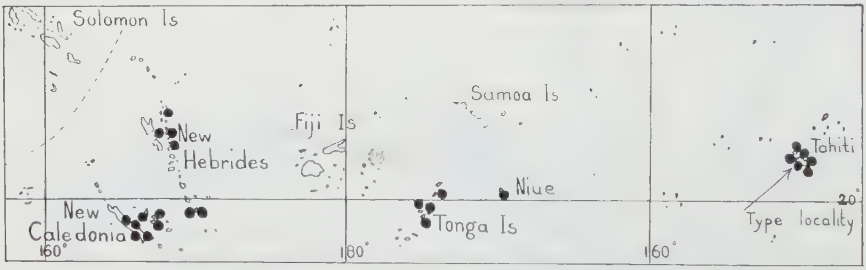
A shrub or a small to medium sized tree up to 15 m. high; branches nearly glabrous or woolly pubescent or canescent and then often soon glabrescent. Stipules filiform, sometimes somewhat subulate, broadened at the base, (2)4–8 mm. long. Leaves (5)10–13(17)-jugate, mostly with an ellipsoid, or narrowly cupulate, or umbilicate gland closely below or more or less between the lowest pair and more or less between the upper 1–7 pairs of pinnae; petiole and rachis canaliculate, pubescent or nearly glabrous; small stipellae sometimes present at the lower one or two pairs of pinnae; pinnae pubescent or nearly glabrous; leaflets (15)20–35(55) pairs on a pinna, oblong-oblanceolate, ± 3–4 times as long as wide, (3)5–10 mm. long, (1) 1.5–2.5 mm. wide, rounded and unequal-sided at the base, obtuse at the apex, glabrous or glabrescent, margin somewhat ciliate.

Inflorescence capitate, globose, pedunculate, 1–4 together in the leaf-axils towards the top of the branches; peduncle 1–4 cm. long, pubescent or nearly glabrous; involucre mostly distant from the full

Fig. 1. *Prosopis insularum* (Guill.) Bret. subsp. *insularum*. 1: flowering branch (× ½); 2: flower (× 6); 3: pods (× ½); 4: part of longitudinal section of pod with exocarp (ex), mesocarp (me), endocarp (en), loculus (lo), and seed (s) (× 2); 5: seed (× 2); 6: transverse section of seed with albumen (al) and cotyledons (co) (× 3) (1 after Moerenhout s.n.; 2 after Nadeaud 507; 3, 5, and 6 after Yuncker 15108; 4 after Balansa 2455).

grown heads, 2–5-lobed. Bracts peltate, somewhat shorter than the calyx, glabrous. Flowers subsessile, bisexual (sometimes a few male flowers in an inflorescence). Calyx funnel-shaped, short-lobed, 2–2.5 mm. long; tube 3–5 times as long as the lobes; lobes obtuse, at the sinus often with few hairs. Petals somewhat longer than the calyx, oblanceolate, 2.5–3 mm. long, subacute at the top, glabrous. Stamens 4–7 mm. long; anthers topped by a stalked deciduous gland. Pistil up to 7 mm. long, often somewhat longer than the stamens; ovary short-stipitate, oblong, somewhat flattened, glabrous, often with a ventral rib or groove; stigma simple.

Pods 1–20 together, often somewhat falcate, flat, oblong to broadly linear, 3–6 times as long as wide, (6)7–10 cm. long, 1.2–2 cm. wide, short-stipitate at the base, obtuse or truncate and mostly short-apiculate at the apex, glabrous, dark brown or black, septate, margins finally gaping but carpel indehiscent, 8–12(15)-seeded, with a thin 0.7–1 mm. broad margin; valves somewhat bulging over the seeds, exocarp desintegrating in flakes, mesocarp when dry nearly 1 mm. thick. Seeds flattened, oblong, about 1.5 times as long as wide, 5–7 mm. long, 3–4 mm. wide, brown, dull; cotyledons surrounded by a layer of albumen.



Map 1. Distribution of *Prosopis insularum* (Guill.) Bret. subsp. *insularum*.

Lectotype: Tahiti, s.l., Bertero & Moerenhout s.n. (P).

Distribution: Islands of the Pacific, from New Hebrides and New Caledonia to Tahiti (see map 1).

Ecology: In coastal rain forest, or along the seashore above high tide limits on rocky and sandy places and there often forming thickets.

Note: Guillemin stated in his original description that the leaflets are "apice brevissime mucronulatis". The leaflets of the lectotype and of all specimens I saw, however, are not mucronulate.

Specimens examined

New Caledonia: near the mouth of the Ouailou R. (or Ouculou R.), Balansa 2455 (P); Poeio et Buobondo, Deplanche 38 (P); s.l., Deplanche 346 (P); s.l., Vieillard & Lancher s.n. (P).

Is. Loyauté: Lifu, Cape Daussy, Balansa 2455*a* (P); Lifu, Däniker 1958*a* (Z); Uvea, Däniker 1958 (Z).

New Hebrides: Pentecost I., Ilamre, Aubert de la Rüe 380 (L; P); Pentecost I., bet. Metaruk and Sasanadam, Aubert de la Rüe 400 (P); bet. Shaik (?) Bay & Hog Harbour, Baker 166 (BM); Vanua Lava I., Kajewski 438 (P); Aneityum I.,

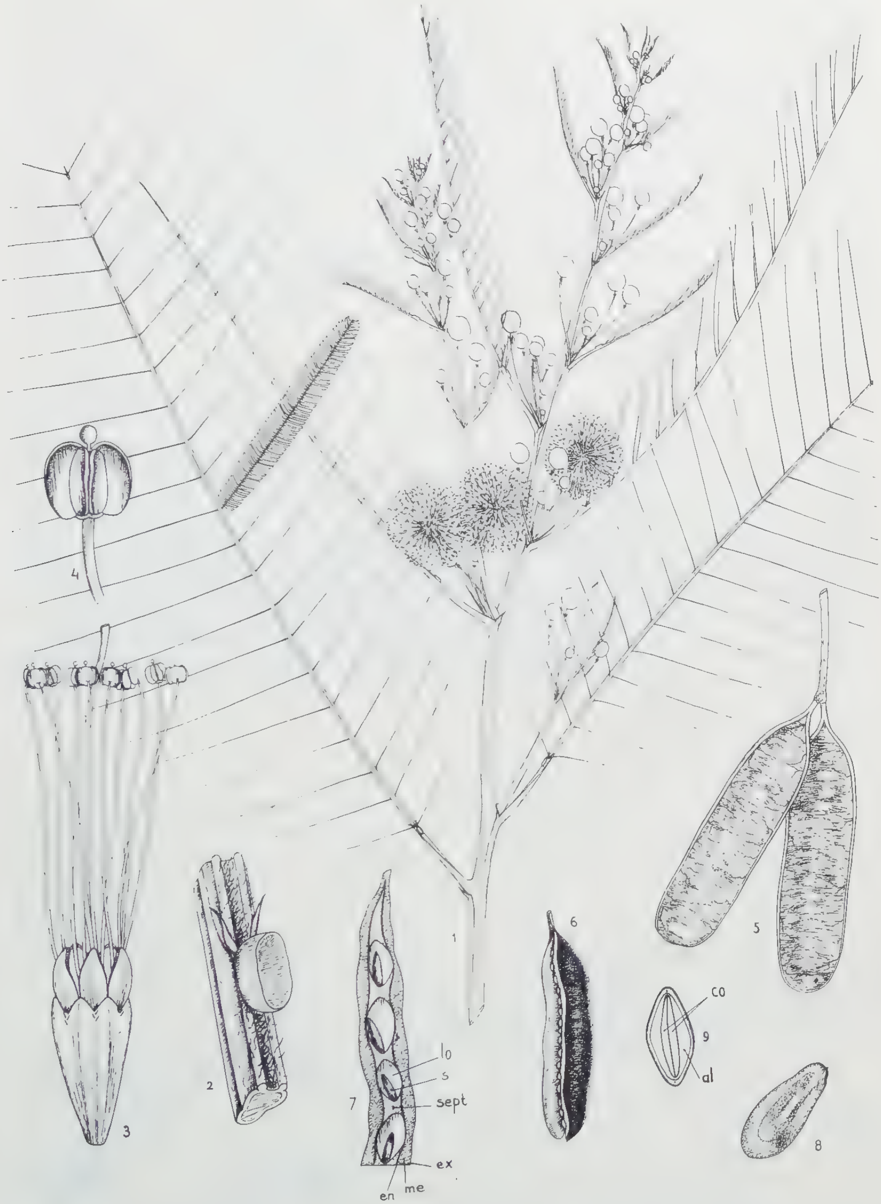


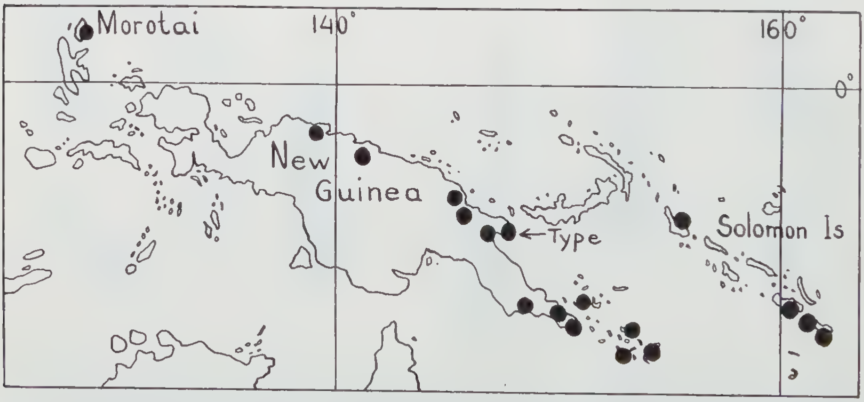
Fig. 2. *Prosopis insularum* (Guill.) Bret. subsp. *novo-guineensis* (Warb.) Bret. 1: flowering branch (× ½); 2: part of the petiole with gland and stipellae (× 5); 3: flower (× 10); 4: anther with gland (× 30); 5: pods (× ½); 6: partly dehiscent pod (× ½); 7: part of longitudinal section of pod with exocarp (ex), mesocarp (me), endocarp (en), sept, loculus (lo) and seed (s) (× 3); 8: seed (× 3); 9: transverse section of seed with albumen (al) and cotyledons (co) (× 5) (1–4 and 6 after Brass 28026; 5 and 7–9 after Henty 9887).

Anelgauhat Bay, Kajewski 944 (P; Z); Aneityum I., Anaunoe, Wilson 993 (P).
TONGA Is.: Tongatapu I., Hürlimann 92 (Z); Tongatapu I., Niutoua, Yuncker 15108 (U); Eua I., Lokupo, Yuncker 15532 (U); Lifuka I., Yuncker 15791 (U).
NIUE: s.l., Jensen 12 (BM).
SOCIETY Is.: Tahiti, Banks & Solander s.n. (BM; P); Tahiti, Bertero & Moerenhout s.n. (lectotype: P); Tahiti, Moerenhout s.n. (P); Tahiti, Nadeaud 507 (P); Tahiti, Vesco s.n. (L; P); Moorea, Taiaraba, Lepine 22 (?) (P).

——— subsp. **novo-guineensis** (Warb.) Bret. comb. et stat. nov. Fig. 2.
Piptadenia novo-guineensis Warburg in Engler, Bot. Jahrb. **13**: 453. 1891; Rechinger in Denkschr. Akad. Wissensch. **89**: 577. 1914; White in Journ. Arn. Arb. **10**: 219. 1929; Merrill & Perry in Journ. Arn. Arb. **23**: 396. 1942.
Schleinitzia microphylla Warburg, l.c.: 336.

A spreading, small to medium sized tree, up to 20 m. high, with a brown longitudinally fissured bark with horizontally streched, pale brown pustular lenticels. Leaves (13)17–24(30)-jugate. Leaflets (25)40–50(65) pairs on a pinna, 2–5(6) mm. long, 0.5–1(2) mm. wide; stipellae mostly present at the lower 1–3 pairs of pinnae.
Flowerheads (1)4–8 together. Calyx 1–2 mm. long. Corolla nearly 1.5 times as long as the calyx, white (?). Stamens purple(?), 4–6.5 mm. long; anthers yellow, with a deciduous gland on top.
Pods straight, (4.5)6–7.5(9.5) cm. long, 1.4–2 cm. wide, truncate or retuse (sometimes short-apiculate) at the apex, (12)15–20-seeded. Seeds 1.5–3 times as long as wide, (4)5–6 mm. long, 2–3 mm. wide, brown or black, glossy.

Type: New Guinea, Finschhafen, Hollrung 598 (n.v.).
Distribution: Malesia, from the Moluccas to the Solomon Islands (see map 2).



Map 2. Distribution of *Prosopis insularum* (Guill.) Bret. subsp. *novo-guineensis* (Warb.) Bret.

Ecology: Secondary rain forest at low elevations or in savannah.
Notes: I could not trace the type specimen of *Piptadenia novo-*

guineensis. In addition to the discussion of the differences between WARBURG's description and mine (see introduction) it may be observed that WARBURG stated that only five anthers were glandulate. He declared that this was an important character for proposing the new genus *Schleinitzia*. The specimens I saw had all anthers glandulate. These glands, however, are early decideous, and therefore it is conceivable that WARBURG found only five anthers with glands.

According to WARBURG the style is much shorter than the stamens. This is seen in young flowers, but at anthesis the style grows and mostly becomes longer than the stamens.

Specimens examined

MOLUCCAS: Morotai, Tobele subdistr., Daigila Penins., Tangkiliaan (exp. Kostermans) 18 (L).

NETHERLANDS NEW GUINEA: Hollandia Res., Depapre, Lam/Versteegh BW 750 (L).

AUSTRALIAN NEW GUINEA: Terr. of New Guinea: Morobe Distr., Lae, Henty 9887 (L); Madang Distr., Gogol Valley, Hoogland 4895 (L); Stephansort, Lewandowsky 29 (L); Toricelli Mt., Schlechter 14598 (P); Papua: Dowara R., Brass 1596 (P); Milne Bay Distr., Cape Vogel Penins., Hoogland 4366 (L); Cape Vogel Penins., N.W. Kwareibo, Saunders 92 (L); Fergusson I., Deidei, Gomwa Bay, Brass 27339 (L); Misima I., Narian, Brass 27569 (L); Sudest I. (Tagula I.), Rambuso, Brass 28026 (L); Rossel I., Abaleti, Brass 28341 (L).

SOLOMON ISLANDS: Bougainville I., Kieta, Kajewski 1594 (L; P); Guadalcanal I., Point Cruz, Walker & White 120 (L); San Cristobal I., Balego-Nagonago, Brass 2698 (L); San Cristobal I., Magoha R., Brass 2736 (L).

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NOTES ON MYRTACEAE IX

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(*received July 28th, 1960*)

I. A PRELIMINARY KEY TO THE SPECIES OF SYZYGIUM IN CENTRAL AND EAST AFRICA

1*a* Leaves cordate at the base, sessile or petiolate. Twigs as a rule sharply quadrangular (except in nr. 2). 2

b Leaves petiolate, acute, obtuse or rounded at the base. Flowers white. 5

2*a* Leaves as a rule not more than 10 cm long, often much smaller, subsessile. Flowerbuds not more than 3 mm in diameter. Flowers white. . . . . 1. **cordatum**

b Leaves and flowers larger, the leaves 8–30 cm long, the adult flowerbuds 5–7 mm in diameter. 3

3*a* Flowers (filaments) white. Inflorescence distinctly branched. Twigs conspicuously winged. 4

b Flowers (filaments) red. Leaves subsessile. Inflorescence short and dense. Twigs at least partly subterete. . . . . 2. **Giorgii**

4*a* Leaves petiolate. Flowers in bud about 5 mm in diameter . . . . 3. **Germainii**

b Leaves subsessile. Flowers in bud about 7 mm in diameter. . . . . 4. **Gilletii**

5*a* A geopyrophyte, emitting short annual flowering stems from a woody base, rarely more than 60 cm high. . . . . 5. **huillense**

b Shrubs or trees. 6

6*a* Calyx-lobes distinct, about 1 mm high, subimbricate in the bud, as a rule persistent and conspicuous on the fruit. Leaves obovate or oblanceolate, mostly rounded and shortly acuminate at the apex. . . . . . . 6. **congolense**

b Calyx-lobes very small. 7

7*a* Flowers very small, the buds scarcely 1 mm in diameter. (Uluguru mountains). . . . . 7. **parvulum**

b Flowers larger, the buds at least 2 mm in diameter. . . . 8

8*a* Twigs quadrangular. Leaves and flowers relatively small, the leaves not more than 9 cm long, as a rule. [1]) 9

b Twigs terete. 10

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[1]) **Syzygium Rowlandii** Sprague, with quadrangular twigs and larger flowers and leaves, is thus far only known from West Africa and Angola.

9*a* Leaves short- or rather long-acuminate at the apex, narrowly elliptic or obovate-elliptic, mostly thin-coriaceous. Mountains of West Africa and the Belgian Congo. . . . . 8. **Staudtii**

b Leaves rigid-coriaceous, broadly elliptic, obtuse or rounded at the apex. Mountains of Tanganyika. . . . . 9. **sclerophyllum**

10*a* Leaves obtuse or with short recurved acumen, the tip easily breaking off. Twigs robust. Montane species. Kivu-district. Ruanda-Urundi. . . . . 10. **parvifolium**

b Leaves as a rule distinctly acuminate, the tip persisting and not recurved. (Some savanna or coastal forms of *S. guineense* (Willd.) DC have also obtuse leaves; their leaves are however larger, 7–15 cm long instead of ± 7 cm long). 11

11*a* Leaves with narrow long acute tip, ovate or lanceolate, broadest below the middle, often rounded at the base. Inflorescence terminal. A swamp tree, according to some collectors with stilt roots or pneumatophores (*S. elegans* Verm. ined.) . . . . 11. **owariense**

b Leaves broadest near or above the middle, with shorter and broader acumen. 12

12*a* Berries oblong, curved. Inflorescence lateral. A cultivated species introduced from tropical Asia, often naturalized, in East Africa possibly also native (*S. polyneuron* Verm. ined.). . . . . 12. **Cumini**

b Berries globose or ellipsoid, not curved. Inflorescence terminal or in some forms lateral. Native species. . . . . 13. **guineense**

1. **Syzygium cordatum** Hochst. ex Sonder in Harvey and Sonder, Flora capensis II (1862) p. 521.

Distribution: Natal, Gazaland, Transvaal, Rhodesia, Moçambique, Tanganyika, the Belgian Congo and Angola. One of the commonest species.

2. **Syzygium Giorgii** De Wildem. in Bull. Jard. Bot. Bruxelles IV (1914) p. 376.

Endemic in the Belgian Congo, as far as known. During flowering, the tree must be rather conspicuous by its red flowers close together in short dense inflorescences. As type specimen must be regarded De Giorgii 1322 (BR). Locally not uncommon.

3. **Syzygium Germainii** Amshoff in Acta Botanica Neerl. 8 (1958) p. 54.

Belgian Congo, Boenda, Tsuaga, Germain 8357 fl. 16 1954 (BR., type, WA); ibidem, Evrard 4021 fl. fr. (BR).

The berry collected by Evrard, though still green and unripe, has probably reached already its full dimensions; it is globose and about 3 cm in diameter.

4. **Syzygium Gilletii** De Wildem. in Bull. Jard. Bot. Bruxelles IV (1914) p. 376.

Belgian Congo: Ravin de Kimpasa, Gillet s.n., typus (BR); rive gauche de la Tsuapa, en face de Mondombe, Evrard 4405 (BR, WA); Route Bikoro - Ingondo, forêt inondable à Mitragyne, Evrard 2680 (BR). Oubangui-Chari: collector unknown (P).

Gaboon: dans la circumscription de Wolen-Ntem, Le Testu 9207 (P).

The collections of Evrard, carefully annotated, make it possible to give a better definition of *S. Gilletii* De Wildem., and to separate it on better characters from *S. Giorgii* De Wildem. (see key). Two of the specimens cited by De Wildeman in his original descriptions under *S. Gilletii* De Wildem., Claessens 311 (BR) and Van De Rijst 2026 (BR) belong, as shown by their inflorescences and red filaments (the colour is still visible in Claessens 311) to *S. Giorgii*. The twigs in *S. Giorgii* are often partly quadrangular, though less strongly and uniformly than in *S. Gilletii*. *S. Gilletii* seems to be less common in the Belgian Congo than *S. Giorgii*, but extends into Gaboon and Oubangui-Chari.

5. **Syzygium huillense** (Hiern) Engl. in Engler Bot. Jahrb. 54 (1917) p. 339; Milne-Redhead in Kew Bulletin 1947 p. 24; *Eugenia guineensis* (Willd.) Baill. ex Laws. var. *huillensis* Hiern, Cat. Welw. Afr. Plants (1898) p. 359.

An undershrub, typically developed as a geopyrophyte emitting annual flowering stems from a woody base, but possibly reaching higher dimensions (i.e. higher than 60 cm) under more favorable conditions, when there is no annual burning. Considered by White ined. as a subspecies of *S. guineense* (Willd.) DC., and in Rhodesia apparently intergrading with *S. guineense* (Willd.) DC. var. *macrocarpon* Engl.

The type is Welwitsch 4401 (BM, LISC) from Huilla, Angola, described as a dwarfshrub, 20–50 cm high, very gregarious and quasi-caespitose, growing on bushy pastures. The two other numbers Welwitsch 4402 and 4403, cited by Hiern, apparently do not belong here; they are described as small trees.

Distribution: Angola, Rhodesia, Belgian Congo, Tanganyika.

6. **Syzygium congolense** Vermoesen n. sp.

Arbor glaber, ramulis brunneis haud quadrangularibus, sed cum lineis distinctis a basi petiolorum decurrentibus. Folia obovata vel oblanceolata, apice plerumque rotundata et abrupte breviter acuminata, basi longe cuneata, 8–12 cm longa, 3–5 cm lata, vel interdum minora, vix discoloria, costa supra impressa, subtus crassa prominente, nervis lateralibus numerosis, tenuibus, utrinque prominulis, sub angulo fere recto abeuntibus, vel parum obliquis. Petiolus gracilis, circ. 1 cm longus. Inflorescentia terminalis, ramulis quadrangularibus, rigidis; floribus ultimis ternis, sessilibus. Alabastra adulta 5 mm

longa, 2–3 mm in diametro, obconoidea, lobis calycis bene evolutis, rotundatis, leviter imbricatis, 1–$1^1/_2$ mm longis. Stylus 7 mm longus; stamina breviora. Bacca globosa, sepalis conspicuis persistentibus coronata, in sicco circ. 1 cm in diametro.

Belgian Congo: Zobia, Claessens 540, type (BR); Yangambi, Germain 8281 (BR, WA); Eala, Pynaert 1166, 849 (BR); Nouvelle Anvers, De Giorgii 597 (BR); Terr. Boende, Tsuapa R., Evrard 3900 (BR, WA); Terr. Walikale, Léonard 1692 (BR, WA), etc.

Uganda: locality?, Dümmer 3236 (U.S., BM, K); Entebbe, Eggeling 4410 (K, BR), cited in Eggeling and Dale, Indigenous trees Uganda (1951) p. 274 under S. guineense (Willd.) DC., etc.

Oubangui-Chari: Thollon 33 (K).

French Cameroons: Forêt d'Essong,? Letouzey 1865 (WA, on dry soil!).

Gaboon: Ngounge, Le Testu 5730 (BM).

A swamp-forest tree, characterized by its well developed sepals and by the form of its leaves. *S. Staudtii* (Engl.) Mildbraed can be confused with this species, but grows in other localities, has quadrangular twigs, shorter sepals and as a rule smaller leaves. I am following the late Vermoesen in describing this as a species, not as a subspecies or variety of *S. guineense* (Willd.) DC.

S. congolense Verm. is apparently widely distributed in aequatorial Africa. A sterile specimen collected by Bancroft (nr. 188) in the Bukoba district of Tanganyika may also belong here; according to the collector it is the same species as that common on the shores of Lake Victoria and its islands in Uganda.

7. **Syzygium parvulum** Mildbraed in Notizblatt XIV (1938) p. 107; Amshoff in Acta Botanica Neerl. 8 (1958) p. 54.

Endemic in the Uluguru mountains of Tanganyika. The type is Schlieben 3922 (BM; BR). Recently, the species has been collected in the same mountains by Drummond and Hemsley nr. 1615 and by Semser nr. 1220. Two of these specimens are densely beset by mosses and lichens.

8. **Syzygium Staudtii** (Engl.) Mildbraed, Wissensch. Ergebn. Deutsch Zentr. Afr. Exp. (1910–1911) p. 188 (1922); Keay in Kew Bulletin 1953 p. 288; *Syzygium guineense* (Willd.) DC var. *Staudtii* Engl. t.c. (1913) p. 582; *Syzygium marounzense* Pellegr. in Bull. Mus. Hist. Nat. Paris (1923) p. 269.

Distribution: French Guinea, Liberia, Ivory Coast, British and French Cameroons, French and Belgian Congo, Gaboon.

A montane species, growing in forests at altitudes of 1000–2400 m or higher still. Sometimes also at lower altitudes. The type is apparently Staudt 491, from the Johann Albrechtshohe, at only 300 m altitude, British Cameroons. The name was however only published by Engler after the same form was recollected by Mildbraed in the Belgian Congo, between Beni and Irumu.

9. **Syzygium sclerophyllum** Brenan in Kew Bulletin 1949 p. 79. Only known from the mountains of Tanganyika.

10. **Syzygium parvifolium** (Engl.) Mildbraed, Wissensch. Ergebn. Deutsch. Zentr. Afr. Exp. (1907–1908) II Bot. 1914 p. 623; Lebrun, Essences forestières Congo Orient. (1935) p. 173 fig. 17; *Syzygium guineense* (Willd.) DC. var. *parvifolium* Engl. in Mildbraed t.c. p. 582.

In the last years, repeatedly recollected in Ruanda-Urundi and also on the other side of Lake Kivu. Not known so far from the other side of the frontier, though very similar to *S. sclerophyllum* Brenan from the Tanganyika mountains (with quadrangular twigs). The type, Mildbraed 1032, is probably lost; in that case, Robijns 2360 (BR), compared by Lebrun with the type in 1935 or before has to be designated as the neotype.
Exsiccata: Pierlot 571, 594, 287, Léonard 3418, 3440.

11. **Syzygium owariense** (Beauv.) Benth. in Pl. Nigrit. (1849) p. 359; Keay in Flora West Tropical Africa Rev. part I (1854) p. 240; Aubréville, Fl. For. C.I. ed. 2 (1959) p. 86; *Eugenia owariensis* Beauv., Fl. Owar. 2, 20 t. 70 (1810); *Syzygium guineense* (Willd.) DC var. *palustre* Aubréville, Fl. For. C.I. ed. 1 (1936) Vol. III t. 268 B; *Syzygium elegans* Verm. ined.

It seems that this species is easier recognized in the field than in the herbarium. The flowers are quite similar to those of *S. guineense* (Willd.) DC., (more glomerate perhaps), the difference in the form of the leaves is not always quite pronounced. While *S. guineense* (Willd.) DC var. *guineense* grows as a rule in fringing forests, *S. owariense* is a swamp-forest tree with (always?) stilt roots or pneumatophores (not known, apparently, in *S. guineense*). The form was first recognized by Aubréville, but needs further study.

Distribution: Sierra Leone, Ivory Coast, Southern Nigeria, Gabon, Belgian Congo (from this region often mentioned as *S. elegans* Verm. ined.); Northern Rhodesia (White ined.); Tanganyika (Iringa-district, Brenan and Greenway 8242; Burrt 6290; Mr and Mrs Hornby 666); Uganda (S. Buddadistrict Dawe 322, 963 (K)).

The name *S. owariense* has often been used for *S. guineense* (Willd.) DC., before a fragment of the type could be studied by Keay who recognized it as the var. *palustre* Aubrév. In Eggeling and Dale, Indigenous trees Uganda the name *S. owariense* is used for *S. guineense* (Willd.) DC var. *macrocarpon* Engl. (p. 275, Dawkins 295 (K)!).

12. **S Cumini** (L.) Alston, a well-known species.

13. **Syzygium guineense** (Willd.) DC.

An extremely variable species. Keay in his revision of the Flora of West Tropical Africa distinguishes the varieties *guineense*, *macrocarpon* Engl. and *littorale* Keay, the latter a coastal form with rather rigid leaves and short petioles, ranging from French Guinea to the estuary of the Congo. White ined. admits for Northern Rhodesia the subspecies *guineense*, *macrocarpon* (a widespread savanna-form), *huillense* (here, until further information, still recognized as a species), *afromontanum* White ined., a mountainform also known from adjacent

Belgian Congo and Angola, and *barotsense* White ined., growing along the upper Zambesi R. These forms show small differences in the form and dimensions of the leaves, in the length of the petioles (very short in *huillense* and *littorale*, often very long in *macrocarpon*), in the dimensions of the inflorescence (lax and manyflowered in *afromontanum*) and in the color of the fruit (said to be red in *barotsense*, grey and not edible in *guineense* var. *guineense*, according to Keay; purple or violet and edible in other forms). The color in the variety *guineense* is, however, probably also purplish to black, according to other collectors. In East Africa there are probably still other forms worthy of recognition.

Syzygium deiningeri Engl. et v. Brehm. in Engl. Bot. Jahrb. 54 (1917) p. 340; Brenan, Checklist Trees and Shrubs Tanganyika Territory Part II (1949) p. 379.

The species seems at the moment only to be known from the description; the type (Tanganyika, Usambaras, Deininger under Holtz 2753 and 2880) was in Berlin and is now lost. Any information about this species will be welcome to the author.

II. A NEW EUGENIA SPECIES FROM THE BELGIAN CONGO

In Bull. Jard. Bot. Bruxelles XXXI (1960) p. 15 Laurent Aké Assi describes a new *Eugenia* species from the Ivory Coast, *Eugenia Miegeana* Ake Assi. However, he cites, except three specimens from the Ivory Coast, among which the type, Aké Assi I A 5295 (a duplicate seen by me in Bruxelles) the same three specimens from Gaboon, which were described by me as *Eugenia gabonensis* Amsh. in Acta Bot. Neerl. 7 (1958) p. 56. Aké Assi describes the flowers also, while I had at the time only seen fruiting specimens, Additional specimens of *E. gabonensis* are: Ivory Coast, Forêt de Yapo, De Wilde 146, 1020 (WA); Forêt de Téké, De Wilde 575 (WA) and the Belgian Congo (poste près de Dimo, Laurent 1072 fl. 11–11–1903 (BR)). Moreover, there is a specimen cultivated in the hothouse of Wageningen raised from seed collected by Dr. de Wit on the Ivory Coast; it was flowering June 1960.

Also, I found during my stay in Bruxelles another African species with cordate leaves, this time from the Belgian Congo, in addition to the three species to which a key is given by Aké Assi.

Eugenia yangambensis Amsh. n.sp.

Frutex 2–5 m altus ramulis teretibus novellis sub lente breviter patente pubescentibus. Folia ovato-lanceolata usque oblonga, apice obtuse acuminata, vel interdum obtusa, glabra, coriacea, (3–) 5–9 cm longa, $2\frac{1}{2}-4\frac{1}{2}$ cm lata; costa supra angusta impressa, subtus prominente, nervis lateralibus circ. 12-jugis, utrinque prominulis, 2–5 mm a margine arcuato-anastomosantibus. Petiolus circ. 2 mm longus, breviter pubescens. Flores fasciculati, axillares vel in nodis defoliatis; pedicelli 2 mm longi, sub lente breviter pubescentes. Sepala 1 mm longa, obtusa; petala 2 mm longa; ovarium fere glabrum. Bacca

rosea, in sicco 8 mm in diametro, monosperma. Embryo homogeneus, curvatus.

Belgian Congo: Yangambi, rive gauche, alt. 470 m, sousbois forêt périodiquement inondée de la Litulumba, le long de la fleuve, Louis 14786 fr. 13–V–1939 (Arbuste hydro-sciaphile de 3 mètres de hauteur. Fleurs nouées. Fruits roses). Yangambi, forêt rivulaire le long du fleuve Congo, Léonard 815 fr. 24–VI–1958 (Arbuste de 5 m de hauteur. Fruits roses). Yangambi, ile Tukutu, alt. 470 m, sousbois forêt primitive lianeuse, Louis 14435 fl. 31–III–1939 (Arbuste sciaphile de 2 metres de hauteur. Fleurs blanches inodores à nombreuses étamines).

By the leaves cordate at the base with the marginal nerve rather distant from the margin this is one of the most distinct species of *Eugenia*, perhaps best comparable with *E. gabonensis* Amsh., with similar but much more elongate, 17–28 cm long leaves. Moreover, *E. gabonensis* seems to grow not higher than 30–80 cm; the specimen in the hothouse of Wageningen was already flowering in its second year.

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ON A GEO-GROWTH REACTION OF THE AVENA COLEOPTILE

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Introduction

In several investigations, particularly in those from the period before the discovery of the auxins, the question has been raised whether the rate of growth of an organ is influenced by its position with regard to the direction of the gravitational force. Is there any measurable difference between the straight growth of an organ in the normal position and that shown on the horizontal clinostat or in the inverse position? Hering (1904) observed that plants which were kept continuously in the inverse position, prematurely end the grand period of growth. Bremekamp (1912) noticed that stems of *Pharbitis*, placed alternately for 24 hours in the normal and in the inverse position, show a reduction of their growth in the latter position.

As to the effect on the growth exercized by a rotation on the clinostat, the results are less uniform. Exact measurements made by Koningsberger (1922) with his auxanometer revealed that the transfer of vertical *Avena* seedlings to the clinostat caused a decrease of the growth rate whereas the seedlings showed an increased growth when they were taken from the horizontal clinostat and were put vertical. Bremekamp (1925) and Dolk (1930, 1936) who in principle obtained the same results, are of opinion that all the seemingly growth accelerations and retardations in reality are due to dorsoventral curvatures of the seedlings. In view of the smallness of the gold plate of the auxanometer, however, the obscuring action of dorsoventral curvatures has been overestimated. They may have accounted for maximally 0.01 percent of the observed effects on the growth rate.

Bara (1957), on the other hand, found an acceleration of the growth of coleoptiles on the horizontal clinostat. Since the speed of revolution of his clinostat was much higher than that of Koningsberger's, this factor may have caused the opposite result. From Larsen's (1953) investigation with *Artemisia* roots we have learned that the speed of revolution of the clinostat may indeed be regarded as a possible cause of contrasting results.

Another obvious comparison was that of the increase in length of a horizontal plant which carries out a geotropic curvature, with that of an upright one which remains straight. According to Sachs (1872), Cholodny (1929) and Navez and Robinson (1933) there would be no difference.

De Wit (1957) compared the rate of curvature of horizontal coleoptiles with that of the back curvature after an opposite geo-induction. He found that the straightening out, followed by a curvature in the opposite direction, is faster than the forwards curvature. The question arises whether the higher rate of back curvature was due to an extra fast growth of the new convex side or to an extra slow growth of the new concave side, or to both. This question, though not studied as such, gave rise to the present investigation, which is concerned with the growth rate of curving and straight-growing coleoptiles in different positions, and in auxin solutions of different concentration.

The main result was that under the conditions of the experiment the geo-growth reaction of the *Avena* coleoptile consists in a reduction of the growth rate in the horizontal position, which is explained by a decreased rate of auxin transport.

Material and methods

The investigation was carried out with isolated coleoptiles from which the primary leaf had been pulled out, and which had all been cut to the same length, viz. of 19 mm. Of these sections the growth rate was studied. The control coleoptiles remained in the normal vertical position, whereas the test plants were treated in one of the following ways:

Treatment A. After the determination of the initial length to tenths of a millimeter, the coleoptiles were placed in a horizontal position. When the geotropic curvature had attained a value between 25 and 35 degrees, the coleoptiles were turned over 180 degrees. On account of the opposite geo-induction received now in the second horizontal position, the coleoptiles straightened out and began to curve in the opposite direction. When the curvatures in the new direction had attained 10 to 20 degrees, the coleoptiles were turned again over 180 degrees and thus brought back in the first horizontal position. As soon as the greater part of the 12 coleoptiles used in one experiment, had straightened out their second curvature, the other coleoptiles which had not yet done so, were smoothly straightened (not stretched) by finger, and the length was compared with that of the controls. The duration of the experiment was 5½–7 hours.

Treatment B. After the determination of the initial length the coleoptiles were placed in a horizontal position. However, in contrast to treatment A, the coleoptiles were prevented from curving geotropically by reversing them every ten minutes. After four hours their length was compared with that of controls which had remained in the normal vertical position during the experimental period.

The first part of the investigation was carried out with non-decapitated coleoptiles in tap water, whereas in the second part the effect was studied with decapitated coleoptiles (2 mm removed) in solutions of indoleacetic acid (IAA) of different strength.

Detailed descriptions of the method and of the apparatus used in

the experiments have been given in previous publications, more especially in ANKER (1954).

RESULTS AND DISCUSSION

The results have been summarized in the Table, which shows in the first place that intact coleoptiles (tip not removed) attain the greatest length in the normal, vertical position; in the second place that it is *not* the bending which reduces the growth of horizontal coleoptiles, since the reduction caused by treatment B was even slightly more pronounced than that caused by treatment A; finally that in decapitated coleoptiles an inhibition of the same magnitude is present in the 0.05 mg/l IAA solution, but that it is absent in the 0.1 mg/l IAA solution.

TABLE

Comparison of the growth of vertical and horizontal coleoptiles

Col. tip	IAA concentr. in mg/l	Treatment	Growth in mm/100 control col.	Growth in mm/100 treated col.	Mean inhibition
Present	0	A	168 ± 8	150 ± 6	
,,	0	A	190 ± 7	167 ± 9	
,,	0	A	273 ± 7	221 ± 9	17 %
,,	0	A	477 ± 20	385 ± 10	
,,	0	A	274 ± 16	222 ± 15	
Present	0	B	133 ± 8	105 ± 6	
,,	0	B	160 ± 3	113 ± 3	21 %
,,	0	B	197 ± 9	169 ± 7	
Removed	0.1	A	260 ± 13	269 ± 12	
,,	0.1	A	304 ± 15	292 ± 15	0 %
,,	0.1	A	265 ± 15	266 ± 15	
Removed	0.2	B	284 ± 9	291 ± 9	
,,	0.1	B	292 ± 13	297 ± 15	— 2 %
,,	0.1	B	268 ± 9	273 ± 6	
Removed	0.05	B	243 ± 8	205 ± 8	
,,	0.05	B	217 ± 14	205 ± 10	
,,	0.05	B	206 ± 12	173 ± 10	14 %
,,	0.05	B	227 ± 8	187 ± 8	

The explanation of the above effect of gravity is not easy, since growth may be influenced in many ways. However, it seems improbable that the inhibition was caused by a decreased auxin production, as the same reduction of growth was observed with decapitated coleoptiles in the 0.05 mg/l IAA solution, in which the vertical and horizontal coleoptiles were supplied with the same quantity of auxin. As a matter of fact, in those instances reported in literature where a change of the auxin production was really found, the lateral action of gravity always caused a stimulation in stead of an inhibition of the auxin production (SCHMITZ, 1933; VAN OVERBEEK *et al.* 1944).

It is further not likely that a change of the reactivity or the sensitivity of the tissue to auxin was involved, since in that case a reduction of the growth should have occurred at both IAA concentrations, since the 0.1 mg/l concentration is still sub-optimal for the growth. For the same reason the idea of a production of growth inhibiting substances may be disregarded.

Finally the inhibition of the growth by gravity could have been effected by a decrease of the rate or the intensity of the transport of auxin; if auxin is added in limiting concentrations, as was done here, a slowing down of the speed of transport must be followed by a decreased rate of growth. With this mechanism the inhibition of intact coleoptiles and that of decapitated ones at the 0.05 mg/l IAA solution can be explained, but the absence of any geo-growth reaction at the 0.1 mg/l IAA concentration, which is still suboptimal (ANKER, 1956), seems to come in conflict with this explanation.

From previous investigations (ANKER, 1956; DE WIT, 1957), we know that IAA permeates through the cuticle, but that in short-lasting experiments (75 minutes) the growth of intact coleoptiles submersed in a 0.1 mg/l IAA solution is not yet measurably influenced. At slightly higher concentrations it is. The possibility, therefore, was investigated, whether in the present, relatively long-lasting experiments, a penetration of IAA from the 0.1 mg/l solution through the cuticle could have obscured effects due to differences in the auxin transport from the cut surface to the base. In three separate experiments of 4½, 5½ and 6 hours duration an increase of the growth was indeed observed; the growth in the IAA solution proved to be 19, 25 and 21 percent respectively higher than that of the controls in water. By this result a serious objection against the idea of a gravity-controlled rate of auxin transport has been removed. As in the earlier experiments of ANKER (1956) and DE WIT (1957), this stimulation was not yet visible after an immersion of 75 minutes.

Measurements of small differences in auxin transport are extremely difficult. They were carried out by PFAELTZER (1934), who obtained variable results, and by VAN DER WEY (1932), whose results provide some support to the transport hypothesis, as a slight decrease of the auxin transport was observed with the *Avena* coleoptile in the inverse position. However, we are in need of a direct proof that gravity affects the auxin transport, as several gravity-induced phenomena are easily explainable by this mechanism. Among the latter are the geo-growth reactions of the *Avena* seedling studied by KONINGSBERGER (1922). The present investigation carried out with relatively small, isolated coleoptile sections, in which dorsoventral curvatures are absent, confirms the results of Koningsberger, and supports the correctness of his interpretation.

The possibility to explain other phenomena by a gravity-controlled rate of auxin transport has been discussed in the Chapter "Orthogeotropism in shoots and coleoptiles" in: Encyclopedia of Plant Physiology Vol. 17II, yet to appear.

SUMMARY

The growth of *Avena* coleoptiles during a period of 5½–7 hours in which they carry out two geotropic curvatures (in opposite directions), and finally are allowed to straighten out, remains behind that of coleoptiles in the normal vertical position. This is true for non-decapitated coleoptiles in water, and for decapitated ones in a 0.05 mg/l IAA solution.

The decreased growth of curving coleoptiles is not due to the bending, since the same reduction is observed when horizontal coleoptiles are prevented from curving geotropically by reversing them every ten minutes.

The above retardation was not found in the 0.1 mg/l IAA solution.

A gravity-induced decrease of the auxin transport is proposed as a possible explanation of the retarded growth of horizontal coleoptiles.

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BOOK REVIEWS

OF PUBLICATIONS RELATED TO BOTANICAL WORK IN THE NETHERLANDS

SCHULZ, J. P.: Ecological studies on rain forest in northern Suriname. Verh. Kon. Ned. Akad. Wet. Afd. Natuurk. 2e reeks, 53 (1).1 –267. 1960, Guilders 17.50, also as: Vegetation of Suriname Vol. II, and: Med. Bot. Mus. & Herb. Utrecht no. 163.

This book, submitted as thesis in 1959, is the result of intensive field work during three years in the forests of Suriname. A general description of the investigated areas is given in the introduction. Part one deals with the results of numerous microclimatological observations made at various levels in parallel series in the mesophytic or rain forest and in large openings; besides a number of readings were taken in an adjacent drier vegetation type, the savanna forest. After a survey of the general climate the following factors are treated in their daily and seasona- variation: light intensity measured with a spherical electric photometer, athmosl pheric humidity recorded with hygrographs with psychrometer readings as controls, evaporation by Piche evaporimeters, air temperature recorded with thermographs and checked by standard and max-min. thermometer readings, and soil temperature by soil thermometers. As a result of the lucky circumstance that the main dry season of 1957 was exceptionally long and severe the measurements cover almost the whole range of climatic conditions in the investigated region.

The author has paid much attention to the ways of both physical and mathematical treatment of his observations and to the value which can be attributed to the calculated figures. Also comparisons have been drawn with data published from other tropical countries.

In part two the soils of the investigated areas are discussed. Along selected transects pits have been dug and additional auger borings made up to 4.5 m deep. Descriptions of the soil profiles are given. Samples of representative profiles have been analysed for mechanical composition in subfractions, acidity, humus content, amount of nutritive ions, and spectre of heavy minerals.

In general a close correlation exists between soil and vegetation, and in some cases the distribution of a tree species appeared to correlate with certain soil characters.

In the dry and wet season a number of constant-volume samples were taken to determine the variation in the percentages of free air space and water at various depths, as aeration and available moisture are of prime importance for the vegetation.

Part three gives a survey of the floristic composition of various types of mesophytic forest in the lowland and in the hills. To this end 17 sample plots of 0.3–1 ha were studied by recording per quadrat of 10 by 10 m all trees of 5 cm diam. and over. Undergrowth of 2–4½ cm was enumerated in 10 percent of the quadrats. For one sample plot of 1 ha in the area of main activity the complete list of species is published with numbers and quadrat frequency in upper, middle, and lower storey, and in the undergrowth. For the other plots only the figures of the more

frequent species are given. At the end comparisons have been made with rain forests in neighbouring countries.

In part four preliminary results of regeneration experiments in the forest are offered. First the diameter-class representation of a number of important timber trees has been determined in various stands and germination and distribution of their seedlings studied. Periodic measurements of the girth of marked trees are still going on, but the first data about girth increment could be inserted. Also attention has been paid to the horizontal distribution of timber trees and to succession in natural and artificial openings in the forest. Remarks on a mixed-forest refining experiment which is under way conclude this part.

The work ends with summaries in English and Spanish. It is well illustrated with maps, diagrams, and 4 full-page photographs.

J. C. LINDEMAN

CHAPMAN, V. J.: Salt marshes and salt deserts of the world. Plant Science Monographs, edited by N. Polunin; London, Leonard Hill (Books) Ltd.; New York, Interscience Publ. Inc., 1960, XVI + 392 pp., 102 fig., 46 Pl., 95 S. net.

CHAPMAN is the first who gives a worldwide survey of the vegetation of salt marshes and salt deserts. No other author would be able to give a better survey. Chapman, professor of botany at Auckland University, is an acknowledged and respected authority on salt marshes, having spent over thirty years in their study and research in many parts of the world. Most of his work has centred on the salt marshes of Great Britain; further studies have been carried out on American salt marshes on both the Atlantic and Pacific coasts. Scandinavian and Dutch salt marshes, and tropical haline flats of Ceylon have also been visited. Since 1946 the writer has been working on salt marshes and mangrove swamps of New Zealand. Finally we must not forget his work on the ecology of marine algae. Therefore CHAPMAN's book is not only a compilation of the results of other workers but presents many of the writer's own results and experiences.

The author divides his subject-matter into 12 chapters: (1) Distribution and characteristics; (2) Physiography and development; (3) Tides and water table; (4) The soil factor; (5) British salt marshes I; (6) British salt marshes II; (7) Arctic and continental European salt marshes; (8) Mediterranean and Australasian salt marshes: Eurasian salt deserts; (9) New World salt marshes; (10) Salt marsh survey and marsh fucoids; (11) Physiology of halophytes and (12) Economic uses.

In our opinion, however, the chapters dealing with environment (2, 3 and 4) are not properly balanced. Chapter 3 includes a great deal of data on the genesis and the mechanical analysis of the soil which might better have been placed in the chapters two and four, respectively. The brief discussion on climatic influences (in chapter 4) suggests a gap in our knowledge of salt-marsh ecology. It is striking that not a single ecologist cited dealing with the rate of sedimentation and the age of the marshes has taken into account the process recently known as maturation of the soil or initial soil formation, which, a.o., results in loss of water and shrinking of the soil-layers. This process which begins soon after the depositing of the silt and mainly depends on the granular composition, on the level on which the soil-layer is situated with respect to tidal fluctuations, and on the drainage, may have a considerable influence upon the writer's results.

From the North and West European salt marshes the list of cited sources might have been extended with papers by REGEL (Kola Peninsula), LIBBERT, PREUSS, VODERBERG and KORNAS (Baltic coasts of Germany and Poland), STERNER and ENGLUND (east coast of Sweden), LEIVISKÄ (coasts of the Botnic Gulf), WESTHOFF (Dutch Wadden Isles), HOCQUETTE and DE LITARDIÈRE & MALCUIT (Belgium and North West France), LEMÉE (Normandy), CORILLON (Brittany), GUINEA and FONTES (west coast of Spain and Portugal).

Classifying the communities, Chapman attemps to bridge the gap between Anglo-American and French-Swiss conceptions by proposing a new system based upon (1) the dominance of species and genera, (2) the Anglo-American concepts of dynamic and stable status of the community belonging to seral or to climax vegetation respectively, and (3) a distinction in relatively extensive communities from those occupying smaller areas.

However commendable Chapman's efforts may be, needless to say that this proposal will not satisfy the phytocoenologists of the French-Swiss School of BRAUN–BLANQUET. With TÜXEN we must draw a distinction between typification and classification. Vegetation types can be described and characterized but not entirely defined and delimited. Classification should be based on that typification and we should bear in mind that each taxon has a syntaxonomic as well as an ecological value. The traditional principle of Braun-Blanquet, the fidelity of taxa, may be, if necessary, supplemented by other criteria, e.g. dominance and vitality.

However, the above remarks will not be able to devaluate this book and are not intended to do so. The work has been written with ardour and great affection for the subject. The phytocoenologist and ecologist will find in it many valuable data and suggestions for both environmental, ecologic, and floristic research. The book includes numerous tables, figures, and photographs. A circumstantial subject and author index makes it handy for reference. Writer, editor and printer have cooperated willingly in making it pleasant and easy to read. The volumes which the writer announces dealing with the mangrove swamps and with the sand dune vegetation of the world are eagerly awaited.

W. G. BEEFTINK

FOTT, Prof. Dr. Bohuslav: Algenkunde, 1959. VII + 482 pp., 17 × 24 cm, 255 fig. in the text and 1 frontispiece. VEB Gustav Fischer Verlag. Half cloth. 48,90 DM.

Again a handbook on algae has appeared, and this time in the German language. The author, who is Director of the Botanical Institute of the Biological Faculty of the "Karls-Universität" at Prague, explains that the book, though primarily written for the use of university students, has been extended to a handbook for the use of scientists working in research and industry. It is essential for those who are working with freshwater algae in all fields. It grips the reader with many original thoughts, as well as compiling thoroughly which is also desirable in a work like this. After each chapter references are given, restricted to the titles of hand- and textbooks (where further references are to be found), and of the articles by those authors who are mentioned in the text.

The book is amply illustrated. Among the numerous figures taken from other authors, there are a number of fine original drawings. The photographs of J. FIALA are of a high quality.

The reason which makes the book of so much interest to students of freshwater algae is that the author, having worked with unicellular freshwater algae so much himself, has given a large place to those groups in all chapters.

In the longest chapter the phyla belonging to the algae are enumerated: *Cyanophyta*, *Chrysophyta*, *Phaeophyta*, *Rhodophyta*, *Chlorophyta*, *Euglenophyta* and *Pyrrhophyta*. Flagellates of doubtful affinity conclude the enumeration. We learn about the cytology, the morphology, the reproduction, the nutrition, the ecology and the geographical distribution of all these phyla. These data are well explained and up to date. Usually one or some of the species belonging to the genera mentioned are enumerated together with morphological, biological or ecological details of interest. Fossil finds are mentioned. Phylogenetical conclusions and remarks are to be found all through the book, which gives room to many individual views. The author regrets that the delineation of the taxa are highly subjective, and that it is not at all defined in the International Code of Nomenclature (this book may be praised for that !). May we not expect that, after thorough monographical investigations, this delineation, at least for the species, may become less subjective? Though mentioned in the references the latest monograph on part of the *Cyanophyta* has not been taken into consideration. For this reason the author could state concerning *Coelosphaerium* Nägeli 1849: "Eine Revision der 9 bisher beschriebenen Arten ist sehr notwendig" (21 species of *Coelosphaerium* have been described and 5 species were transferred to that genus from other genera). *Chaetomorpha linum* (Müll.) Kütz. and *Ch. ærea* Kütz. are considered by the author two separate species, though T. Christensen (Bot. Tidsskr. 53, 1957, 311–316) has proved that both belong to one and the same species.

In another chapter the ecology and habitats of the algae are amply treated. This chapter is a most interesting, very complete and skilfully composed account of the subjects mentioned, containing many individual finds and thoughts. It deals with: plankton, neuston, benthos, aerophytic, soil and thermal algae, algae living on snow and ice, in salt water, on plants and animals, and with symbiosis and parasitism.

There is a chapter dealing with the use of algae, which is only possible in the case of large quantities. The fresh water algae are well considered also here. Valuable information can be obtained about the relation between fishes and algae (food, poisoning), which is important for pisciculture, about the quality (cleanness) of the water, about algae as indicators in biological analyses of waters, algae as manure and as food for men. However, it seems amazing to find *Ulva lactuca* mentioned sought after for salad everywhere where it occurs, and especially in the Mediterranean region. The industrial and medical uses of algae are amply dealt with.

J. Th. K.

INDEX

New taxa and new combinations in **bold face**